

# International PhD Program in Neuropharmacology

## XXVII Cycle

### **Anti-inflammatory and antioxidant activities of Nrf2/HO-1 activators: *in vitro* studies in microglia and retinal cells**

PhD Thesis

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## LIST OF ABBREVIATIONS

Nrf2	Nuclear factor (erythroid-derived 2)-like 2
HO-1	Heme Oxygenase-1
DMF	Dimethyl fumarate
INF- $\gamma$	Interferon- $\gamma$
LPS	Lipopolysaccharide
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
PGE2	Prostaglandin E2
shRNA	small hairpin RNA
NG	Normal Glucose
HG	High Glucose
ROS	Reactive Oxygen Species
ARE	Antioxidant Response Element
Keap1	Kelch-like ECH-associated protein 1
CO	Carbon Monoxide
CRP	Cytochrome P450
BVR	Biliverdin Reductase
CO-RMs	CO-Releasing Molecules
NF- $\kappa$ B	Nuclear Factor-kappa B
AP-1	Activating Protein-1
HYCOs	Hybrid CO-releasing molecules
BACH1	BTB And CNC Homology 1, Basic Leucine Zipper Transcription Factor 1
CDDO	2-Cyano-3,12-Dioxooleana-1,9(11)-Dien-28-Oic acid
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-CoA

FBS	Fetal Bovine Serum
CNS	Central Nervous System
AD	Alzheimer's Disease
PD	Parkinson's Disease
NO	Nitric Oxide
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
IL-1	Interleukin-1
IL-10	Interleukin-10
TFG- $\beta$	Transforming Growth Factor- $\beta$
iNOS	Inducible NO Synthase
COX-2	Cyclooxygenase-2
IL-4	Interleukin-4
BSO	Buthionine-S-Sulfoximine
MPP+	1-Methyl-4-PhenylPyridinium
GSH	Reduced glutathione
GSSG	Oxidized glutathione
mtDNA	mitochondrial DNA
OCR	Oxygen Consumption Rate
ECAR	ExtraCellular Acidification Rate

## **ABSTRACT**

Inflammation and oxidative stress are critical components of the cellular response to various types of stress and play essential signaling roles during the progression and resolution of disease states. However, it is now being recognized that protracted and persistent inflammation and oxidative stress underlie several chronic diseases that affect the Western countries. This is particularly relevant in the ageing population, where neurodegenerative diseases and diabetes, among others, are predominant pathological states that require constant monitoring and pharmacological interventions that target multiple pathways involved in these diseases. Microglia cells, the resident macrophages of the brain, work under normal conditions to protect neurons and surrounding tissue from infectious agents and by removing apoptotic and necrotic cells to promote tissue healing and repair. Similarly, retinal pigmented epithelial cells exert an important supporting role in the eye by protecting photoreceptors and mediating nutrient uptake for retinal tissue. Because of their anatomical position and function, both cell types participate in the pathophysiology of neurodegenerative diseases and retinal diabetes complications.

A highly relevant cellular response to stressful stimuli is mediated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a redox sensitive transcription factor that promotes the up-regulation of several cytoprotective and detoxifying enzymes. Among these genes is heme oxygenase-1 (HO-1), the enzyme responsible for degradation of heme into iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. The three enzymatic products of HO-1 have been shown to possess anti-inflammatory, antioxidant and protective actions in models of disease characterized by redox imbalance and inflammation and lack of HO-1 exacerbates damage and injury in animal experimental models and two human cases reported in the literature. The Nrf2/HO-1 axis is amenable to pharmacological manipulation and the search of active molecules that target these pathways is of high interest for the discovery of therapeutic approaches to counteract neuroinflammation and retinal diseases.

The work presented herein focuses on the characterization of the anti-inflammatory and antioxidant activities induced by activators of the Nrf2/HO-1 axis. In the first instance I identified the most effective Nrf2/HO-1 inducers that modulate the inflammatory response in BV2 murine microglia cells. I searched the literature and selected 56 compounds reported to activate Nrf2 or HO-1 and analysed them for HO-1 induction and cytotoxicity *in vitro*. Approximately 20 compounds up-regulated HO-1 at the concentrations tested (5 to 20  $\mu$ M) with carnosol, supercurcumin, cobalt protoporphyrin-IX and dimethyl fumarate (DMF) exhibiting the best induction/low cytotoxicity profile. Up-regulation of HO-1 by some compounds resulted in increased cellular bilirubin levels which correlated with the potency of the inducers to inhibit nitrite production after challenge with interferon- $\gamma$  (INF- $\gamma$ ) or lipopolysaccharide (LPS). The compounds strongly down-regulated the inflammatory response (TNF- $\alpha$ , PGE<sub>2</sub> and nitrite) in cells challenged with INF- $\gamma$  than LPS, and silencing HO-1 or Nrf2 with shRNA significantly reversed this effect. Based on these interesting findings I chose the Nrf2 inducers DMF and carnosol and investigated their effect on antioxidant pathways, oxygen consumption and wound healing in human retinal pigment epithelial cells (ARPE-19) cultured in medium containing normal (NG, 5 mM) or high (HG, 25 mM) glucose levels to mimic hyperglycemic conditions. I found that Nrf2 activation and heme oxygenase activity increased in ARPE cells treated with 10  $\mu$ M DMF or carnosol irrespective of glucose levels. However, HG rendered retinal cells more sensitive to regulators of glutathione synthesis and decreased both cellular and mitochondrial reactive oxygen species. Further analysis revealed that culture in HG progressively decreased respiration and ATP over time with evident morphologically damaged mitochondria. Acute treatment with DMF or carnosol did not restore oxygen consumption in HG cells. Interestingly, using the scratch assay *in vitro* I found that wound closure was faster in cells cultured in HG than NG and was accelerated by carnosol. This effect was reversed by an inhibitor of heme oxygenase activity, supporting a pro-healing role of HO-1.

In summary, I show that some small activators of Nrf2/HO-1 are effective modulators of microglia inflammation and exert interesting pharmacological activities in retinal cells cultured under normal or hyperglycemic conditions. Thus, these findings highlight promising chemical



scaffolds that can serve for the synthesis of potent new derivatives to counteract neuroinflammation and retinal diseases.

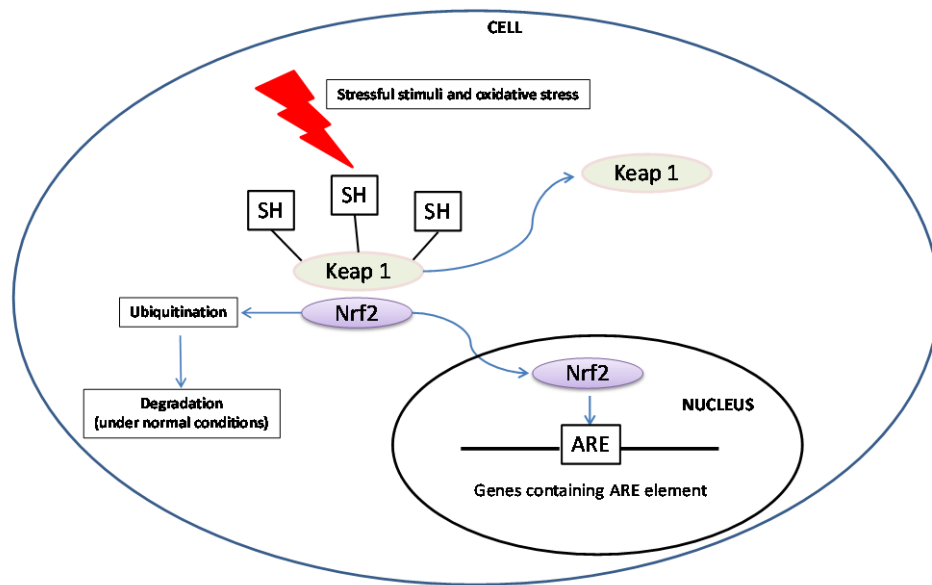
## GENERAL INTRODUCTION

### THE Nrf2/HO-1 AXIS

#### The transcription factor Nrf2

Cellular oxidative stress derives from an imbalance between excessive production of reactive oxygen species (ROS) and an inadequate antioxidant defense, leading to damage of macromolecules such as proteins, lipids and DNA [1]. It is generally accepted that oxidative stress initiates and contributes to the progression of many diseases. To maintain redox homeostasis in physiological conditions cells rely on an array of antioxidant systems, such as glutathione, superoxide dismutase and catalase [2]. However, when challenged with oxidative stress cells have evolved sophisticated and potent defensive responses that are controlled by the transcriptional factor Nrf2 [3-5]. Nrf2 is currently considered the master regulator of the antioxidant response via its binding to the antioxidant response element (ARE) of target genes that encode for cytoprotective and detoxifying enzymes. The biochemical mechanism responsible for Nrf2 activation has been elucidated, showing that Nrf2 is retained in the cytosol by its repressor protein Keap1 under normal conditions. The Nrf2-Keap1 complex maintains low Nrf2 levels by promoting Nrf2 ubiquitination and degradation by the proteasome [5,6]. Following stressful stimuli this 'silent form' of Nrf2 is liberated via detachment from Keap1 and its subsequent accumulation in the nucleus causes induction of ARE-dependent genes [7]. The modification of highly reactive cysteine residues present on Keap1 and essential for Keap1-Nrf2 interaction is central to this activation process (**Figure 1**) [8,9]. Genes containing ARE in their promoter include HO-1, glutathione S-transferase, NAD(P)H:quinone oxidoreductases, glutamylcysteine ligases, thioredoxins and others, representing a coordinated response that neutralizes ROS and oxidants and re-establishes a healthy cellular environment [5,7]. Nrf2

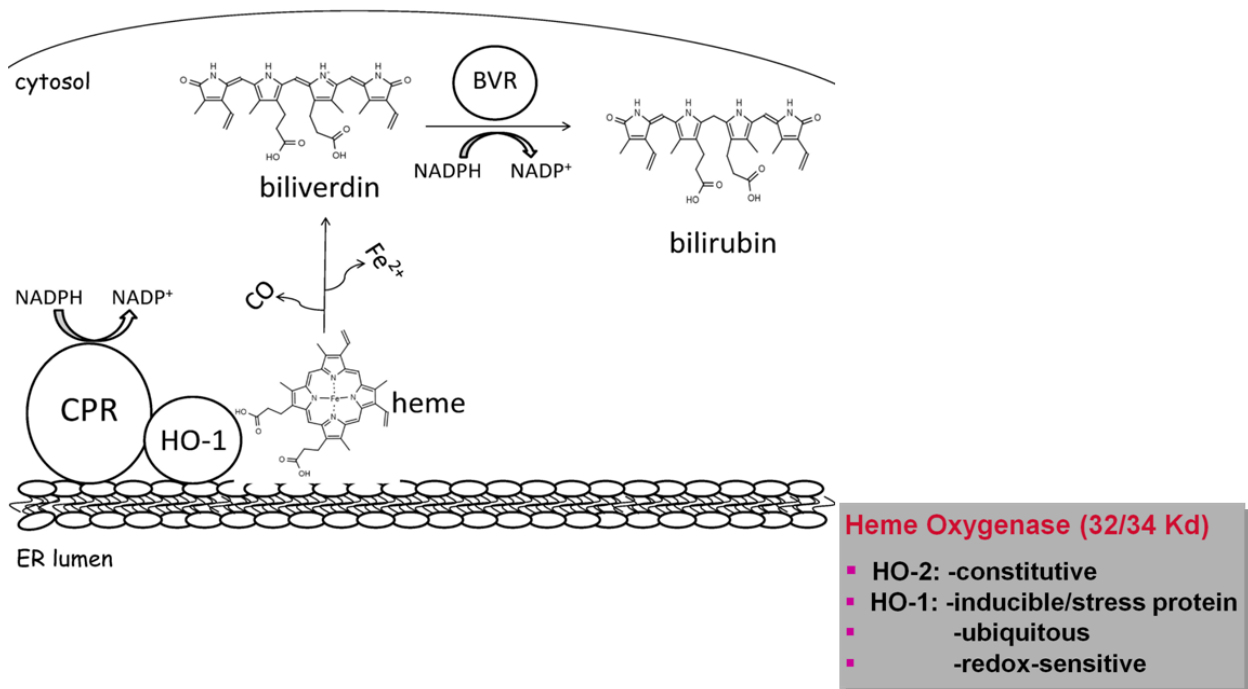
deficient mice are more susceptible to oxidative stress damage [10] and silencing of Nrf2-dependent genes reverses to a significant extent the beneficial activities of Nrf2 activation [11-13]. Interestingly, Nrf2 also plays a significant protective role against inflammation, an effect that may be associated with an interdependence of ROS as signaling molecules for the stimulation of the inflammatory response [14]. Innamorato and coworkers demonstrated in Nrf2 deficient mice an increased production of inflammatory molecules and microglia activation in the brain after endotoxin challenge [15]. Pulmonary genes typical of the of the pro-inflammatory and innate immune response were augmented to a higher extent in Nrf2 null than wild type mice after endotoxic shock caused by cecal ligation and puncture [12]. Protective actions of Nrf2 have been described in many other models of disease, including neurodegenerative diseases, and these findings in general highlight the ability of tissues to mount an endogenous and intrinsic defensive response that could be exploited for therapeutic purposes [16-22].



**Figure 1.** Activation of Nrf2 in the cell.

## Heme oxygenase and tissue protection

One relevant enzymatic system dependent on Nrf2 is HO-1, the inducible isoform of heme oxygenase which can be up-regulated in all tissues upon many kinds of stress [23-25]. The heme oxygenase pathway is an essential cellular defense system that has been maintained throughout evolution, being present in algae and progressing in time to mammalian organisms [26,27]. Its role as an effective antioxidant is quite particular, since it does not, like the classic antioxidant enzymes catalase or superoxide dismutase, transform a toxic oxidant (hydrogen peroxide or superoxide anion) into a molecule harmless for cells. In fact, heme oxygenase exerts its antioxidant function by removing a pro-oxidant molecule, heme, while simultaneously producing metabolites that are endowed with unique protective characteristics, i.e. carbon monoxide (CO) and the couple biliverdin/bilirubin (**Figure 2**) [25,28-30]. Iron is also released during heme degradation by heme oxygenase and its increased intracellular levels lead to up-regulation of ferritin, an iron-storing protein that participates to the cytoprotective machinery



**Figure 2.** The heme oxygenase enzymatic pathway. CRP, cytochrome P450; BVR, biliverdin reductase.

engaged by heme oxygenase to combat stress conditions [31]. Due to capacity of heme oxygenase-derived products to modulate many fundamental cellular functions, it is perhaps too restrictive to label this enzyme as an antioxidant and it is probably more appropriate to consider it as a regulator of homeostasis. This is exceptionally true for HO-1, which serves a dual purpose as ‘sensor/effector’ by sensing cellular stress (oxidative, nitrosative, inflammatory and metabolic) and damage and efficiently attempting to rescue tissue viability and functions [30,32-35]. These properties might also be linked to a recently identified truncated form of HO-1, which can translocate into the nucleus and activate oxidant-responsive transcription factors [36]. The obligatory role this pathway plays in the preservation of tissue integrity is crucially exemplified in human and murine HO-1 deficiency, which both exhibit increased oxidative stress, persistent vascular injury and chronic inflammation [37,38].

Heme oxygenase-derived products contribute to protection by modulating many cellular processes. For example, CO possesses vasorelaxing properties, prevents systemic and pulmonary hypertension and displays remarkable anti-inflammatory and anti-bacterial effects [39-41]. Important findings from different pre-clinical experimental models of disease have demonstrated that administration of CO gas at doses that are well tolerated in animals alleviates inflammatory processes and vascular disorders and protect tissues against ischemic injury [25,42-45]. These unexpected and promising results triggered the idea of utilizing CO as a therapeutic expedient [25] notwithstanding the notion that inhalation of CO gas and the delivery of precise amounts of CO gas to living organisms is not an easy task and caution needs to be taken to avoid undesired toxic effects [46,47]. CO gas is now being investigated for its therapeutic properties and clinical trials are ongoing to evaluate whether CO inhalation is beneficial against pulmonary hypertension, organ transplantation and other pathologies ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). An alternative way to deliver CO as a pharmacological agent is via CO-releasing molecules (CO-RMs), a novel class of compounds that can be viewed as a ‘solid form’ of CO [48-54]. CO-RMs are essentially transition metal carbonyls which can transfer small amounts of CO to cells and tissues and are now used as a tool to study the role of CO in biology; their potential therapeutic features have been confirmed *in vivo* in rodents and pigs where the molecules showed efficacy against inflammatory conditions such as sepsis and rheumatoid

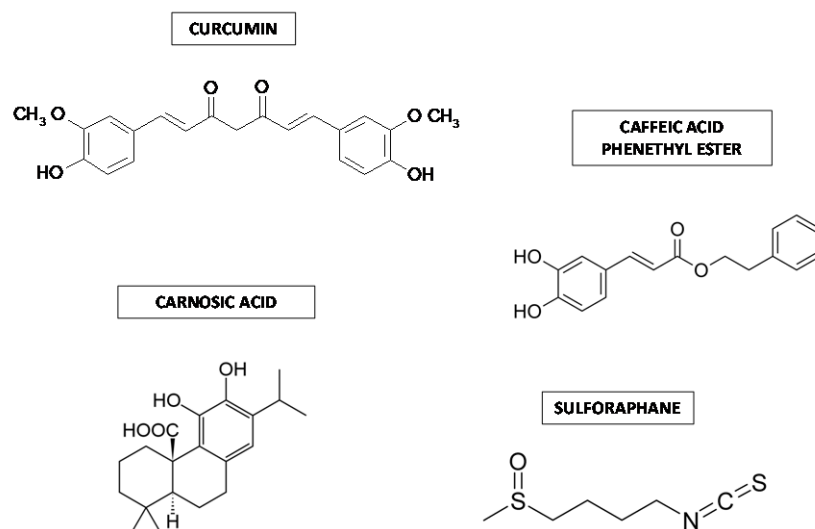
arthritis [55-59] and protected organs from reperfusion injury [49,60,61], bacterial infection [62,63] and arterial thrombosis [64].

The other products of HO-1, biliverdin and bilirubin, possess remarkable antioxidant properties and participate in immunomodulatory processes of cells and tissues [65-68]. In a seminal work by Stocker and colleagues these linear tetrapyrroles were originally demonstrated to neutralize oxidation of membranes *in vitro* with a capacity higher than vitamin C, which is regarded as the best antioxidant against lipid peroxidation [65]. These findings were confirmed by demonstrating that HO-1-derived bilirubin protects vascular smooth muscle cells and cardiac tissue against oxidative stress and ischemia-reperfusion injury [66-68]. Bile pigments have also been shown lately to protect against vascular injury and inflammation in animal disease models of vasculopathy, thrombosis and allograft rejection [69-71]. From a therapeutic perspective, the beneficial properties of bilirubin are underscored by studies in human subjects reporting a lower prevalence of vascular complications in diabetic patients with Gilbert syndrome, a condition characterized by hyperbilirubinemia [72]. Notably, these subjects displayed decreased levels of glycosylated hemoglobin, reduced markers of oxidative stress and inflammation, and improved lipid profiles [72]. As in the case of CO, the protective mechanism(s) of biliverdin and bilirubin needs to be further elucidated. However, one great advantage is that these metabolites are endogenously produced; that is, they are generated by heme oxygenase in proximity of cellular components where oxidation and damage take place, thus potentially being more effective in cellular protection compared to classical antioxidants, which are exogenously introduced with the diet. Therefore, an effective pharmacological approach aimed at enhancing the endogenous production of HO-1 metabolites would be to design and synthesize novel pharmaceuticals acting as potent inducers of HO-1. There exists a growing number of molecules, such as plant-derived polyphenols but also pharmaceutical compounds like probucol, that possess the ability to up-regulate HO-1 in tissues [30,73-76]. These findings are interesting because they highlight that HO-1 protein expression can be manipulated by exogenously applied substances thus representing a pharmacologically-responsive target.

## Targeting the Nrf2/HO-1 axis for drug discovery

Nrf2 is currently the focus for the development of novel therapeutic approaches, with specific molecules, such as small synthetic triterpenoids and dimethyl fumarate (DMF) [17,77-79], and broccoli sprout extract rich in sulforaphane, a well-known Nrf2 activator [80], being investigated in clinical trials following a series of positive results in pre-clinical studies. Indeed, the recent approval (2013) by the Food and Drug Administration of DMF as a drug for the treatment of multiple sclerosis (commercial name: Tecdifera) exemplifies this trend. The promising beneficial effects of targeting Nrf2 have also been surprisingly demonstrated in autistic subjects. The study demonstrated that participants affected by autism and receiving capsules of sulforaphane-rich broccoli sprout extracts on a daily basis improved behavior, social interaction and verbal communication compared to subjects taking placebo [81]. The idea of using Nrf2 as a drug target shows how the field of drug discovery is currently moving from the concept that one agent should act as an agonist (or antagonist) of a particular protein or enzyme, thus inhibiting or activating its activity, versus the synthesis of molecules that affect one pathway (i.e. Nrf2) responsible of multiple pleiotropic and protective activities dependent on up-regulation of downstream genes.

To act as Nrf2 activators chemical entities have to possess electrophilic properties, i.e. they have to exhibit in their chemical structure electron-deficient carbon centers that react with nucleophiles (electron rich) [82,83]. Protein thiols or sulfhydryl groups, such as those found in glutathione, are typical nucleophiles. Keap1, the repressor of Nrf2, contains 27 cysteine residues and some of them are critical for the reaction with electrophilic molecules that allows for the release of Nrf2 from the Keap1/Nrf2 complex and translocation of this factor into the nucleus [8]. It is interesting to observe that several compounds derived from plants (curcumin, caffeic acid phenethyl ester, chalcones) are electrophilic or pro-electrophilic, that is, they become active electrophiles upon oxidation (for example, carnosic acid) [73,74,82,84,85]. **Figure 3** shows the chemical structure of known Nrf2 activators with electrophilic properties.



**Figure 3.** Chemical structure of Nrf2 activators

The induction of HO-1 in different cell types and tissues has been studied for many years independently of Nrf2. Several compounds, including the substrate heme, heavy metals like zinc, cadmium and cobalt, have long been recognized for their ability to increase HO-1 [86]. However, it was only when curcumin, a component of curry found in the South Asian root *Curcuma longa*, was discovered by serendipity to strongly enhance HO-1 expression in endothelial cells and renal proximal tubule cells, that the first prototype of a small exogenous molecule acting as inducer of HO-1 was identified. Significantly, in both studies by Motterlini's group in endothelial cells and by Agarwal's group in renal cells, curcumin was employed either as an inhibitor of the nuclear factor-kappa B (NF- $\kappa$ B) or the activating protein-1 (AP-1) transcription factors, in an attempt to elucidate whether these molecular mechanisms affected HO-1 induction under hypoxic conditions and inflammation, respectively [73,87]. Both groups were surprised to find that, instead of suppressing heme oxygenase, curcumin was by itself a potent inducer of HO-1. These interesting findings stimulated a search for novel molecules with similar properties and it was then demonstrated that caffeic acid phenethyl ester, rosmolic acid,

and chalcones could all increase HO-1 to different extents in various cells [74,84,88]. It was also shown that Nrf2 was fundamental in curcumin-mediated HO-1 induction and structural-activity relationship studies revealed that the  $\alpha,\beta$ -unsaturated carbonyl functionalities of these chemicals are necessary for the activation of the HO-1 and thus for eliciting cytoprotective and anti-inflammatory action [74,89,90]. In the last few years many new compounds of natural origin have been reported to similarly affect HO-1 and this list will probably keep growing [91]. However, we have recently performed a screening of compounds reported in the literature as Nrf2 activators/HO-1 inducers with interesting results (CHAPTER I). In this study we have assessed HO-1 protein expression by ELISA in BV2 microglia cells exposed to low micromolar ranges (5-20  $\mu$ M) of 56 small molecules and found that the original substances identified as HO-1 inducers, including curcumin and carnosol, were still the most potent HO-1 activators exhibiting good HO-1 expression/low toxicity profiles. These data suggest that certain chemical scaffolds are perhaps unique and 'evolutionarily' selected to specifically activate this pathway. Moreover, the existence of such compounds provides us with a variety of templates and chemical scaffolds that can be explored for the design of novel drug-like molecules. One of the latest developments in this direction has been the design and synthesis of hybrid molecules termed 'HYCOs', from **Hybrid CO**-releasing molecules. Starting from a CO-releasing compound and a chemical scaffold exhibiting electrophilic characteristics, a new molecule that simultaneously activates Nrf2 and liberates CO was produced [92]. HYCO-1 potently induced nuclear accumulation of Nrf2 and HO-1 expression in different cell lines and markedly decreased endotoxin-mediated nitric oxide accumulation, an index on inflammation. This effect was stronger than that obtained with the Nrf2 activator or the CO-releasing molecule alone, indicating that this dual activity molecule may be useful for optimizing the protective properties of the Nrf2/HO-1/CO pathway.

From a broader biological perspective, it is fascinating that a group of plant-derived substances, mostly representing secondary metabolites synthesized during environmental stresses such as lack of nutrients, disease or infection, also up-regulate HO-1 and Nrf2. That is, molecules produced in response to stress and to confer stress tolerance in plants are also capable to



induce pathways that increase resistance to stress in animal tissues, emphasizing the conservative approach of nature throughout evolution.

That HO-1 expression is regulated by additional nuclear proteins and transcriptional factors other than Nrf2 should be considered. In fact, it has also been reported that a repressor, the BTB and CNC homology 1 basic leucine zipper transcription factor 1 (Bach1), controls HO-1 expression [93]. Bach1 is a heme-binding nuclear protein which represses the HO-1 gene in normal physiological conditions but is displaced to free the HO-1 promoter upon stress and when intracellular heme levels rise. The authors of this important discovery also showed that alleviation of repression by Bach1, rather than activation of Nrf2, is critical for HO-1 induction as mice lacking Bach1 constitutively expressed high HO-1 levels in most organs. Interestingly, examination of the chromatin structure of HO-1 indicates that it is in a pre-activation state under normal conditions, with Bach1 acting as a repressor but rapidly sensing environmental cues that result in transcription of HO-1. This phenomenon is quite interesting because it suggests that cells possess a dynamic, rapid and extremely sensitive system with the explicit task to respond to endogenous stressful changes and that HO-1 is up-regulated as one of the first effector molecules to convey these signals. Therefore, if manipulation of endogenous HO-1 expression is envisaged for therapy approaches, small molecule activators of this pathway must, at least, have dual effect by de-repressing Bach1 and activating Nrf2.

### **Aspecificity of HO-1 inducers and the issue of heme availability as a substrate of HO-1 enzymic activity**

A variety of pathways may be affected by the compounds that are called HO-1 inducers. The very fact that to promote HO-1 induction most of these substances act on transcriptional modulators up-stream of HO-1 indicates that other genes may well be stimulated/down-regulated at the same time. Whether this is a disadvantage is, however, debatable. In fact, if HO-1 inducers can actually affect positively other defensive systems and possibly suppress negative or deleterious cellular signals independently of HO-1 it is a good thing. Curcumin is a classic example of a HO-1 inducer with multiple molecular targets that affect cellular processes related to inflammation, tumorigenesis, apoptosis and possibly many others to be discovered.

Synthetic triterpenoids are another example, being small electrophilic molecules that activate Nrf2 and HO-1 and are under investigation for a variety of inflammatory conditions. Yore and colleagues have reported that exposure of HEK293 and PC-3 cells to the triterpenoid CDDO-Imidazolide changes the expression of 577 proteins involved in hormone and insulin sensing and other important signal transduction pathways, exposing the multifunctional properties of these compounds which may contribute to their mechanism(s) of action [94] but also to their side-effects. However, any drug, whether already commercialized or in development will influence multiple pathways in addition to the one being developed for; statins are inhibitors of cholesterol synthesis by blocking 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, show pleiotropic activities and, incidentally, also induce HO-1 [95]. Perhaps the focus should be, together with designing targeted approaches, to attempt to reduce as much as possible the unwanted and health-damaging effects of molecules that show promising features.

An important issue to consider in the case of HO-1 is heme availability. It is not known whether the increased HO-1 protein following gene induction by an exogenous stimulator will have access to enough heme to be converted to the protective products. There would be little use for higher levels of HO-1 if the substrate is limited, unless HO-1 is beneficial for reasons other than its enzymatic reaction. We have recently determined whether exposure of BV2 microglia cells to different inducers results in accumulation of bilirubin in the culture supernatant. We found that certain substances, including carnosol and curcumin, concomitantly stimulated HO-1 expression and bilirubin production while others elicited only HO-1 induction (CHAPTER I). In addition, it appeared that the source of heme was external, i.e. the fetal bovine serum (FBS) with its residual haemoglobin content (18.5 mg/100 ml according to the certificate of analysis of the fetal bovine FBS used in our cell culture work) present in the culture medium. These findings are puzzling and we have not examined if they are particularly relevant for microglia cells or also for other cell types (for example cardiomyocytes, which have high heme content). However, one can envision inflammatory situations, such as hemorrhagic stroke, in which microglia cells will have access to abundant heme/haemoglobin and therefore be able to quickly degrade them if HO-1 is up-regulated. A similar scenario might occur in other pathological conditions and different tissues. Thus, when working on the potential use of HO-1

inducers for drug discovery, it would seem essential to establish with accurate methods their capacity to also increase heme oxygenase-derived products in tissues.

## MICROGLIA AND NEUROINFLAMMATION

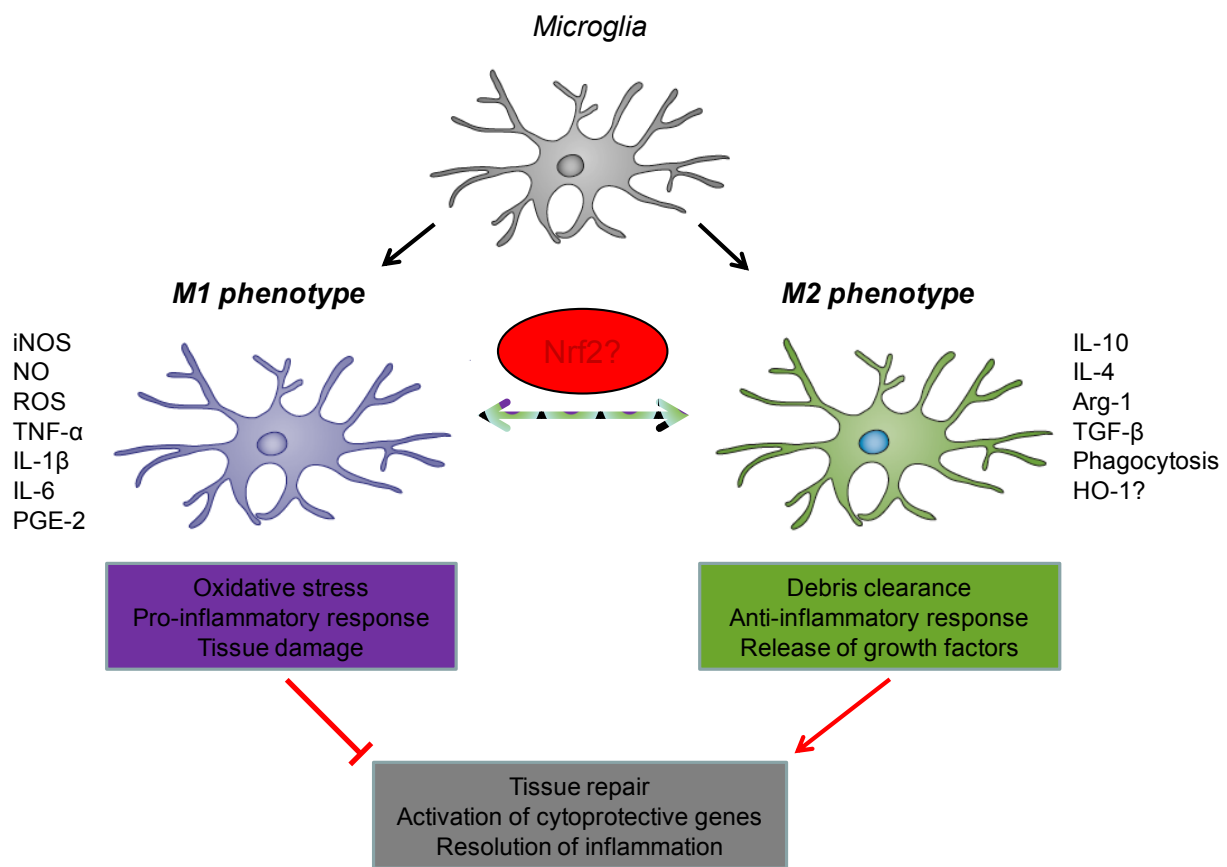
### Microglia in neurodegenerative diseases

Persistent inflammation and oxidative stress in the central nervous system (CNS) are key processes in the progressive decline of neuronal activities that characterize a number of neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, glaucoma and retinal neurodegeneration [96,97]. The initiation and propagation of neuroinflammation rely very much on the interaction between glia, immune cells and neurons although microglia are central to this response as they are the unique resident immune cells of the CNS [97]. Microglia are specialized “macrophages of the brain” that are distinguished from other glial cells, such as astrocytes and oligodendrocytes, by their origin, morphology, gene expression pattern and functions [98,99]. Although present in all brain regions, microglia are mainly found in the grey matter achieving the highest concentration in hippocampus, olfactory telencephalon, basal ganglia and *substantia nigra*, and comprise between 0.5-16% of total nerve cells [100]. The factors that drive these differences in tissue distribution of microglia are not well-understood, but it is important to note that species differences also exist; in particular, the numbers of microglia are significantly higher in white matter areas of the human CNS than in the corresponding rodent tissues [101]. Microglia possess the necessary elements and features to act as sensors of disruption in normal homeostasis within the mature CNS. Even small disturbances of homeostasis in the CNS microenvironment can trigger activation of microglia, a process that results in morphological changes and up-regulation of a diverse array of genes and intracellular molecules. Activated microglia can have diverse expression profiles, with the capacity to synthesize a broad spectrum of both proinflammatory and anti-inflammatory cytokines and other molecular mediators [97]. On one hand, activation of microglia following an injurious stimulus promotes brain recovery by clearing cell debris, resolving local inflammation

and releasing a plethora of growth factors. On the other hand, microglia can impede the repairing processes while exacerbating tissue damage by releasing pro-oxidants and harmful cytokines. Microglia are rapidly activated following a number of pathologic events including altered neuronal function, infection, oxidative injury, brain ischemia, stroke and systemic inflammation [102]. If protracted for a long period of time, activation of microglia can ultimately result in a chronic inflammatory state that accelerates the progression of neurodegeneration and neuronal injury [98].

Microglia-driven neuroinflammation is associated with a broad spectrum of neurodegenerative pathologies and has been more detailed in Alzheimer's disease (AD) and Parkinson's disease (PD). Accumulation of misfolded  $\beta$ -amyloid-containing proteins and alpha-synuclein, histopathological hallmarks of established AD and PD both in animals models and the brain of AD or PD patients, are frequently associated with increased number of activated microglia in degenerated brain regions [103-106]. In addition, studies with positron emission tomography using a radiotracer that quantifies microglia in AD patients *in vivo* revealed increased levels of activated microglia in brain regions that are affected by the disease [107]. Several inflammatory mediators generated by microglia have been reported to contribute to neuronal damage in neurodegenerative diseases. For instance, increased nitric oxide (NO) and tumor necrosis factor (TNF- $\alpha$ ) production by microglia in response to amyloid-beta has been directly implicated in the neuronal cell cycle events that lead to the development of AD [108]. Similarly, release of proinflammatory cytokines and increased generation of ROS as a consequence of microglial activation have been shown to be associated with neuronal loss in PD [104,109]. The important role of these microglia-derived mediators in the evolution of neuronal damage in PD has been confirmed by showing that suppression of activated microglia prevents neurodegeneration in an animal model of PD [110]. It is interesting to note that a similarity between AD pathology and retinal degenerative diseases such as glaucoma and diabetic retinopathy has been reported pointing to chronic neuroinflammation as the common denominator of these disorders [111]. For instance, increased levels of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and TNF- $\alpha$  have been found in the vitreous fluid of diabetic patients [112] and retinal TNF- $\alpha$  levels is increased in diabetic patients affected by proliferative diabetic retinopathy [113]. The pro-inflammatory

profile that typifies diabetic retinopathy has been recapitulated in animal models of diabetes also exhibiting increased IL-1 $\beta$  [114] and TNF- $\alpha$  [115] levels in the retina. In addition, and similar to the canonical neurodegenerative brain diseases, microglial activation in the retina has been found in human diabetic retinopathy [116] and in animal models of type 2 [117] diabetes. Activated microglia in the CNS are commonly referred to as M1-like or M2-like, in accordance with the distinctions proposed for ‘cytotoxic’ and ‘cytoprotective’ subpopulations of macrophages in other organs (**Figure 5**).



**Figure 5.** Phenotypic polarization of microglia with production of proinflammatory (M1) or anti-inflammatory mediators (M2)

This terminology has been proposed to define the distinct cellular phenotype acquired by microglia and macrophages in response to different microenvironmental cues *in vitro*. Classically activated (M1) microglia and macrophages release destructive proinflammatory mediators such as TNF- $\alpha$ , NO, interleukin-1 (IL-1), ROS and IL-6. By contrast, alternatively activated (M2) microglia and macrophages clear cellular debris through phagocytosis and release numerous protective factors and anti-inflammatory mediators such as arginase-1, interleukin (IL-10) and transforming growth factor- $\beta$  (TFG- $\beta$ ). It is important to emphasize that the distinction between M1 and M2 phenotypes only reflects two extreme activation states of immune cells that have been fully characterized *in vitro* experiments. The status of microglia *in vivo* is more complicated than *in vitro* and most likely involves different populations of cells with overlapping phenotypes and functions. Nonetheless, this classification is extremely useful for understanding the functional status of microglia in brain damage and their important role in progression of neurodegenerative disorders. Although the pathways and the signaling mechanisms involved and regulating the dynamic switch between M1 and M2 remains to be fully characterized, recent evidence suggest that redox homeostasis plays an important role in influencing the acquisition of the final microglia phenotype. In this context, activation of the antioxidant Nrf2/HO-1 axis may significantly contribute to determine the interplay between the pro- and anti-inflammatory status of microglia in neurodegeneration.

### **Nrf2 in the control of microglia phenotypes**

As mentioned above, one of the principal features of activated microglia in addition to their ability to generate pro-inflammatory cytokines is the increased release of ROS such as superoxide anion, hydrogen peroxide and hypochlorous acid as well as reactive nitrogen species including NO and peroxynitrite. These strong and damaging oxidants are produced primarily by NADPH oxidase, myeloperoxidase and inducible NO synthase (iNOS). The consequent changes in the cellular redox status and the signalling pathways activated by this response have important implications for the fate of microglial function and their role in mediating either pro- or anti-inflammatory effects. In fact, emerging data point to an imbalance in the redox status of the cells as the prime signal responsible for the activation of specific

transcription factors that control the change in microglia phenotype. For instance, NADPH-derived ROS have been implicated in the activation of NF- $\kappa$ B, a master regulator of the microglial inflammatory response to brain infections and to environmental and cellular damage [118]. Notably, activation of NF- $\kappa$ B by ROS leads to the transcription of several pro-inflammatory genes such IL-1 $\beta$ , iNOS and TNF- $\alpha$  [118,119]. The connection between redox state and microglial plasticity has been confirmed in vitro and in vivo. Oxidative stress and the inflammatory response induced by the environmental toxin rotenone in microglia enhanced the levels of M1 phenotypic genes (TNF- $\alpha$ , iNOS and cyclooxygenase-2(COX-2)/PGE<sub>2</sub>) but reduced M2 markers such as IL-10 [120]. Pharmacological inhibition of NADPH oxidase or genetic deletion of its functional subunits (p47<sup>phox</sup>) has been shown to change microglia from M1 to M2 phenotype. In this study it was found that mice lacking p47<sup>phox</sup> displayed a lesser amount of microglia in the hippocampus alongside a marked reduction in pro-inflammatory mediators. In contrast, upon challenge with LPS these mice showed an increased mRNA expression of the anti-inflammatory marker interleukin-4 (IL-4) [121]. Thus, it appears that increased oxidative stress in microglia is strongly associated with a pro-inflammatory phenotype. This is also emphasized by the findings that changes in the pool of glutathione, the prime line of defense against oxidative stress in the cell, alter the extent of microglial activation. Treatment of human microglia with buthionine-S-sulfoximine (BSO), which depletes intracellular glutathione, induces oxidative stress and an inflammatory response that causes the cells to secrete TNF- $\alpha$ , IL-6 and NO thus damaging neuroblastoma SH-SY5Y cells in a co-culture model in vitro [122]. Predictably, stimulation of glutathione synthesis had opposite effects. Similarly, increased glutathione synthesis results in less activation of BV2 microglia by LPS and administration of the thiol donor N-acetylcysteine in rats following stroke significantly reduces the expression of pro-inflammatory markers in the brain [123,124]. These studies strongly indicate a direct relationship between oxidative stress signals and the acquisition of a pro-inflammatory profile in activated microglia.

Considering that ROS and reactive nitrogen species must be lowered after the initial pro-inflammatory response to prevent further tissue damage, induction of anti-oxidant and cytoprotective genes can be envisioned as a plausible counter-signal in activated microglia

undergoing M2 polarization, thus acquiring an anti-inflammatory phenotype. This concept has been initially demonstrated in a range of studies performed primarily on macrophages but recent emerging evidence indicates that this scenario is pertinent to microglia as well. As mentioned in the sections above, activation of the Nrf2/HO-1 axis typifies the response of the cell to oxidative stress and inflammation. Interestingly, expression profiling performed on human macrophages polarized to produce TNF- $\alpha$  (M1) or IL-10 (M2) revealed that HO-1 was mostly expressed in M2 macrophages [125]. In addition, M1-polarizing cytokines inhibited while M2-stimulating factors enhanced HO-1 expression. Moreover, inhibition of heme oxygenase activity decreased whereas an inducer of HO-1 increased LPS-triggered IL-10 release from M2 cells suggesting that HO-1 is important for the anti-inflammatory activity of M2-polarized macrophages. Mandal and colleagues reported that adiponectin stimulate M2 polarization in macrophages through the regulation of HO-1 expression and subsequent suppression of LPS-stimulated cytokine production [126]. Induction of HO-1 by hemin significantly attenuated inflammation in hypertensive rats by selectively enhancing the anti-inflammatory macrophage M2 phenotype while suppressing the proinflammatory chemokines released by macrophage M1 phenotype [127]. In a mouse breast cancer model, suppression of HO-1 activity by zinc protoporphyrin IX resulted in tumor associated macrophages in a switch from alternatively (M2) to classically activated macrophages [128]. Genetic ablation of hmox-1 gene in *H. pylori*-infected mice increased histologic gastritis, which was associated with enhanced M1 responses; gastric macrophages of *H. pylori*-infected mice and macrophages infected in vitro with this bacterium showed an M1/Mreg mixed polarization type [129].

In parallel to HO-1, the participation of Nrf2 in the acquisition of microglial phenotype was demonstrated by comparing parameters of inflammation in the hippocampus of NRF2-deficient and wild type mice following systemic treatment with LPS. It was found that lack of Nrf2 leads to exacerbation of neuroinflammation induced by LPS, as determined by an increase in M1 typical markers (F4/80, iNOS, IL-6, and TNF- $\alpha$ ) compared to wild type mice. In line with these observations, activation of Nrf2 by sulforaphane significantly reduced the number of microglial cells in the hippocampus and attenuated the production of M1-markers in response to LPS [15].

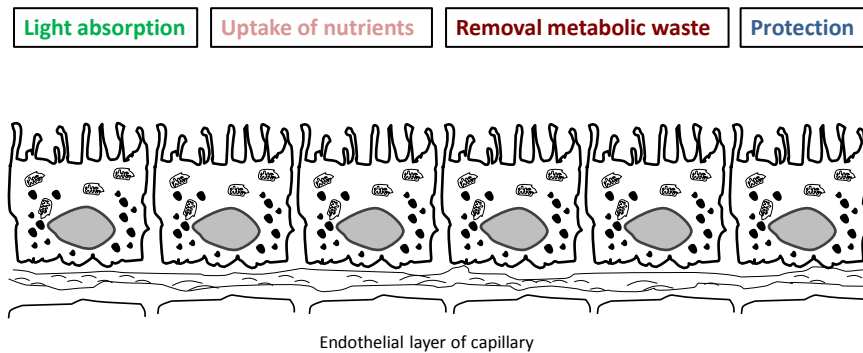


Concerning neurodegenerative disorders, studies conducted in Nrf2-deficient animals pharmacologically treated with 1-methyl-4-phenylpyridinium (MPP+) to induce Parkinson disease exhibited more astrogliosis and microgliosis as determined by an increase in inflammatory markers characteristic of classical microglial activation (COX-2, iNOS, IL-6, and TNF- $\alpha$ ) and a concomitant decrease in anti-inflammatory markers attributable to alternative microglial activation (arginase-1 and IL-4) [130]. These results were confirmed in microglial cultures stimulated with conditioned medium from dopaminergic cells treated with MPP+, further demonstrating a role of Nrf2 in maintaining a balance between classical and alternative microglial activation [130]. Altogether, these studies demonstrate a direct involvement of both HO-1 and Nrf2 in the control of macrophages and microglial dynamics and suggest a crucial role for this antioxidant axis in the modulation of pro- and anti-inflammatory phenotypes. The fact that electrophiles and other naturally derived compounds can modulate NF-kB, Nrf2 and HO-1 supports the role of these substances in the modulation of inflammation extensively described in the literature (see also CHAPTER I). As a consequence, it is likely that electrophiles will regulate the phenotype and polarization of macrophage/microglia cells, although direct investigation of this hypothesis is still very limited in the literature and only a recent new study has reported that curcumin reduced the M1 phenotype and polarized RAW 264.7 macrophages towards the M2 phenotype [131].

## **RETINAL TISSUE AND DIABETES**

### **Retinal pigment epithelium**

The retinal pigment epithelium is a single layer of cells located between the light-sensing photoreceptors and the choriocapillaris of the eye [132-134] and together with the endothelial cells of the retinal vessels they constitute the blood-retinal barrier (**Figure 4**). Because of this anatomical position, the retinal epithelium functions in support and protection of photoreceptors; like other epithelial cells in the body, they are polarized [134].



**Figure 4.** Retinal pigment epithelium layer and summary of main functions (adapted from Strauss O., *Physiol Rev* 85: 845–881, 2005)

Therefore, retinal epithelial cells uptake from the blood nutrients such as glucose, fatty acids and ions and deliver them to the photoreceptors. In addition, they remove metabolic waste for the maintenance of cellular homeostasis. The retinal epithelium is also responsible of the storage of retinoid and its metabolism in a form that can be utilized by photoreceptors. The retinal epithelium is pigmented because it contains several pigments including melatonin, lipofuscin and flavins. By absorbing excessive light, these pigments protect the retina from light-induced toxicity (phototoxicity) [134].

An additional function of the retinal epithelium is to produce and secrete growth factors (vascular endothelial growth factor, pigment epithelium-derived growth factor) important for the functional integrity of the photoreceptors and the underlying endothelium [134]. The retinal epithelium also releases immunoprotective factors that confer immune privilege to the ocular tissue. Damage to retinal tissue, including the retinal epithelium, is associated with the development of diseases including retinitis pigmentosa and age-related macular degeneration, major causes of blindness worldwide [135]. Furthermore, hyperglycemia and diabetes have marked negative effects on the eye, which suffers from delayed wound healing, edema and ulcers [136]. Diabetic retinopathy, a disease where inflammation is thought to play an

important role, is also a cause of blindness for people above 50 years of age and damage to retinal endothelial cells, epithelium and increased vascular permeability with retention of leukocytes are observed in this condition [136].

### **Oxidative stress and retinal tissue damage**

The retina is especially vulnerable to damage caused by oxidative stress. In fact, light exposure and the high oxygen tension in blood of the choriocapillaris are a significant source of oxidative (and nitrosative) stress and represent a significant threat to the health and functional integrity of the retinal epithelium [135,137]. Although abnormalities in retinal function, such as changes in gene expression and markers of cellular senescence together with loss of photoreceptors, may be present in ocular tissue of young subjects, the pathological features of eye diseases are usually manifested in aging individuals (age 60-65) [137,138]. The retinal epithelium possesses, like other tissues, the capacity to respond to oxidative stress and strongly relies on Nrf2 to mount the antioxidant endogenous defense necessary for its survival. In this respect, recent studies demonstrated in Nrf2 deficient mice an age-dependent accumulation in the retinal epithelium of pigments, inflammatory proteins and aggregates connected to the autophagosome and lysosome, similarly to what is observed in the human eye of individuals with age-related macular degeneration [139]. In early works using the Nrf2 activator sulforaphane it was shown that the compound protected a human retinal pigment cell line (ARPE-19) against phototoxicity caused by light-activated all-trans-retinaldehyde [140] and an oxidized pigment [141]. The Nrf2 system has also been shown to be an essential counteractive machinery against retinal damage induced by smoking. In mice lacking Nrf2, smoking induced DNA damage, cellular degeneration and apoptosis of retinal cells which were more pronounced than in wild type animals [142] and treatment with an Nrf2 inducer increased resistance to the deleterious effects of chronic exposure to cigarette smoke [137,143].

Glutathione in retinal cells also contributes to the endogenous antioxidant defense. Its conversion between reduced (GSH) and oxidized form (GSSG) is fundamental in the neutralization of intracellular oxidants and glutathione reductase is the specific enzyme which converts oxidized glutathione into its reduced form [144]. Glutathione-synthesizing enzymes

are also dependent on Nrf2 [5,16]. Interestingly, electrophiles bind to glutathione, leading to a temporal depletion of the antioxidant that is suspected to be involved in the cellular toxic effects of the compounds (CHAPTER II). Satoh et al. [82] have recently argued that electrophiles can be divided in categories based on their modulation of glutathione levels and the activation of the Nrf2-dependent counterattack [82]. Thus, electrophiles in the first category strongly deplete glutathione with a minimal activation of the stress response that leads to protection (e.g. doxorubicin). Electrophiles of the second category exert a milder depletion of glutathione and also activate an electrophilic counterattack, resulting in protection in at least a limited concentration range (e.g. DMF). Electrophiles in the third category strongly activate the Keap1/Nrf2 response without glutathione depletion (e.g. carnosol and carnosic acid); in contrast, together with Nrf2 they also stimulate glutathione synthesis thus exhibiting important therapeutic properties. In other words, if depletion of glutathione prevails over the mounting of the defensive counterattack then the electrophile will increase cellular stress and toxicity. As glutathione protects retinal cells from oxidized retinoids toxicity [145], it is not surprising that sulforaphane was found to reduce cell death mediated by photoreactive pigments [146]. In addition, earlier studies in retinal epithelial cells documented a strong association between the degree of protection elicited by sulforaphane against oxidative stress mediated by menadione and various oxidants and the elevation of glutathione levels [147].

Increased oxidative stress is manifested in high glucose conditions and is thought to be a major abnormality in diabetic retinopathy. Oxidative stress resulting from increased production of ROS was observed after short-term (24 h) exposure of retinal endothelial cells to high glucose conditions and this burst of ROS generation was accompanied by increased HO-1 expression [148]. Castilho and colleagues also reported that inhibition of heme oxygenase activity exacerbated the toxic effects of elevated glucose, the oxidant hydrogen peroxide and excess NO, indicating the protective role of HO-1 in the retinal tissue challenged with hyperglycemia. The authors conclude by suggesting that impairment of the antioxidant response in the retina may be a possible cause of diabetic retinopathy. Conversely, the induction of antioxidant enzymes is expected to protect against this pathology, in line with observations published by Fan and colleagues who reported that HO-1 induction by hemin was associated with decreased

apoptosis and activation of other antioxidant enzymes such as superoxide dismutase 1 and B-cell lymphoma 2 in a rat model of diabetic retinopathy induced by streptozotocin [149]. However, it should be noted that the diabetic environment compromises the antioxidant defense system, with effects on antioxidant enzymes and glutathione levels. Kowluru and colleagues showed in a series of articles that the activities of major antioxidant enzymes, including glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, were decreased in the retina of diabetic rats [150]. Similarly, in the same diabetes model levels of reduced glutathione were diminished while oxidized glutathione was increased [151]. This group has also recently investigated the Nrf2 system in retinal tissue in the context of diabetes and found that, even though Nrf2 expression is increased in the retina of diabetic rats, its signaling properties are decreased because less Nrf2 accumulation was observed in the nucleus and its DNA-binding activity is impaired [152]. Importantly, the study examined also tissue from human donors and confirmed these findings in subjects with diabetic retinopathy.

Thus, damage induced by oxidative stress and diabetes changes the antioxidant profile of retinal tissue and it is possible that the reduced antioxidant protection is part of a cause/effect dynamic, creating, together with the external stressor (oxidants, high glucose levels), a vicious circle for the initiation and progression of retinal diseases. The above observations raise the hypothesis that pharmacological manipulation of Nrf2 may be a useful stratagem to combat retinal damage and vision loss over the long term. A report recently published by Chang and coworkers supports this idea [153]. These authors have developed a model whereby retinal pigment cells derived from induced pluripotent stem cells collected from patients suffering from age-related macular degeneration are used in drug screening. Interestingly, the authors found that the antioxidant response was significantly decreased in these cells and that, among several natural compounds tested, the Nrf2/HO-1 activator curcumin was the most effective in reducing oxidant-mediated cell death and impaired retinal cells function.

### **Mitochondria dysfunction in high glucose-induced retinal damage**

Mitochondria are a major cellular source of ROS both in physiological conditions during oxidative phosphorylation and during pathophysiological states, when early ROS increase is

postulated to be a signaling mechanism that promotes cell adaptation to stress [154]. Mitochondria are also being implicated in sensing chronic stress and one example of this is hyperglycemia, as observed during diabetes, which is a type of 'metabolic' stress exerting profound perturbations of mitochondrial function [155]. This is not surprising, since mitochondria control energy production from the respired oxygen and nutrients and play a central role in the regulation of energy metabolism. Accumulation of mitochondrial damage will affect the cell capacity to generate energy giving rise to cell dysfunction, inflammation and senescence. In this respect, it is tempting to speculate that chronic hyperglycemia leads to accelerated cellular aging and that culture of retinal cells in high glucose conditions may be a useful model to study senescence mechanisms.

It is beginning to be recognized that high glucose negatively influences the function of retinal tissue by targeting mitochondria [156]. For example, it was shown that oxidative stress as a consequence of high glucose conditions or long term diabetes (8 months) in rats led to release of cytochrome C from mitochondria and accumulation of apoptotic proteins in the mitochondria, with resulting apoptosis of retinal cells [157]. In accordance with the fact that mitochondrial DNA (mtDNA) is highly susceptible to oxidative stress, it has been reported that long term diabetes increases mtDNA damage with a consequent decrease in gene expression of proteins of the electron transport chain encoded by mtDNA [158]. A dramatic phenomenon described in the same work was that even after re-establishing good glycemic control with insulin 6 months after the initiation of diabetes with streptozocin, mitochondrial dysfunction was not reversed, suggesting that retinal cells exhibit some kind of 'metabolic memory' and that mitochondria-associated components could be contributing to this effect [158]. Accompanying these findings, it was reported that an impaired mtDNA replication system, controlled by superoxide anion generated in the mitochondria, is observed in diabetic retinal tissue [159]. Therefore, it appears that mitochondrial dysfunction develops in the diabetic retina because of the oxidative stress induced by high glucose levels. The compromised biochemical and molecular parameters measured in the diabetic retina are also reflected in morphological changes of mitochondria, although it is difficult to determine whether the morphological changes are a cause or a consequence of mitochondrial dysfunction. Trudeau et

al have recently examined mitochondrial morphology in retinal endothelial cells and pericytes exposed to high glucose for 6 days and found that mitochondrial fragmentation and membrane potential were increased [160,161]. Similarly, I report in CHAPTER II that retinal pigment epithelial cells exhibit morphological changes consistent with mitochondrial swelling and less defined cristae when cultured in high glucose, as assessed by transmission electron microscopy. This effect was evident as early as 1 week after exposure to high glucose. Apart from an increase in apoptosis, which was observed in retinal endothelial cells grown in high glucose [157,161], the functional consequences of mitochondrial damage mediated by high glucose are expected to affect mitochondrial respiration and energy production. By measuring the oxygen consumption rate of living cells with the XF analyzer (Seahorse Bioscience) it has been in fact demonstrated that oxygen consumption rate (OCR) is decreased in retinal endothelial cells [160], pericytes [161] and epithelial cells (CHAPTER II) cultured for several days in high glucose. This is the first demonstration that high glucose elicits a decreased cellular respiration compared to normal glucose levels and highlights the impaired metabolic activity of retinal cells induced by hyperglycemic conditions. In the case of retinal epithelial cells the lower respiration was matched by a diminished ATP production in high glucose cultured cells, effectively proving the reduced ability of these cells to generate energy (CHAPTER II). It is very interesting that all the three types of retinal cells have been shown to similarly diminish their mitochondrial respiration in the presence of high glucose, confirming the high susceptibility of retinal cells to high glucose-mediated damage which includes mitochondria.

Another notable observation concerns the effect of high glucose on glycolysis in the three cell types. The XF analyzer measures simultaneously oxygen consumption and extracellular acidification rate (ECAR), which is an index of glycolysis. Since mitochondrial respiration was compromised by high glucose, it would be expected that glycolysis is increased to compensate for the decreased energy production as a result of reduced mitochondrial oxidative phosphorylation. This was corroborated in retinal endothelial cells [161] but not in pericytes [160] or epithelial cells (CHAPTER II), which instead displayed a decrease in ECAR. The reason for this divergence is unknown at present, but a difference in glucose transport by these cells could partially explain these results.

The changes in mitochondrial morphology and function in response to hyperglycemic environment indicate that novel therapeutic strategies aimed at preventing retinal damage should focus on preservation of mitochondria. A recent study in retinal epithelial cells demonstrated that incubation with resveratrol for 24 or 48 hours increased mitochondrial respiration and that pretreatment with this compound protected cells against acrolein-induced cytotoxicity and reversed the inhibition of mitochondrial bioenergetics caused by acrolein [162]. Whether Nrf2 activators possess similar pharmacological properties in the context of diabetic-induced mitochondrial dysfunction in the retina remains to be explored.



## CHAPTER I

## Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells

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**List of non-standard abbreviations:** ALAS 1, delta-aminolevulinate synthase 1; ARE, antioxidant responsive element; BVR, biliverdin reductase; tBH, tert-Butyl hydroquinone; CA, carnosol; CAA, carnosic acid; CoPP, Cobalt Protoporphyrin IX; CO-RMs, carbon monoxide-releasing molecules; DHC, 2,2-Dihydroxychalcone; DMF, dimethyl fumarate; H, hemin; HO-1, heme oxygenase-1; Keap-1, Kelch-like ECH-associated protein-1; INF- $\gamma$ , interferon- $\gamma$ ; ISL, isoliquiritigenin; LPS, lipopolysaccharide; Nrf2, HF-E2 p45-related factor 2; PGE2, prostglandin E2; S, sulforaphane; SC, supercurcumin; shRNA, small hairpin RNA; SnPPIX, tin protoporphyrin IX; TNF- $\alpha$ , tumor necrosis factor alpha.

## Abstract

The nuclear factor erythroid derived 2-related factor 2 (Nrf2) and the antioxidant protein heme oxygenase-1 (HO-1) are crucial components of the cellular stress response. These two systems work together to combat oxidative stress and inflammation and are attractive drug targets for counteracting different pathologies, including neuroinflammation. We aimed to identify the most effective Nrf2/HO-1 activators that modulate the inflammatory response in microglia cells. In the present study, we searched the literature and selected 56 compounds reported to activate Nrf2 or HO-1 and analysed them for HO-1 induction at 6 and 24 h and cytotoxicity in BV2 microglial cells *in vitro*. Approximately 20 compounds up-regulated HO-1 at the concentrations tested (5 to 20  $\mu$ M) with carnosol, supercurcumin, cobalt protoporphyrin-IX and dimethyl fumarate exhibiting the best induction/low cytotoxicity profile. Up-regulation of HO-1 by some compounds resulted in increased cellular bilirubin levels but did not augment the expression of proteins involved in heme synthesis (ALAS 1) or biliverdin reductase. Bilirubin production by HO-1 inducers correlated with their potency in inhibiting nitrite production after challenge with interferon- $\gamma$  (INF- $\gamma$ ) or lipopolysaccharide (LPS). The compounds down-regulated the inflammatory response (TNF- $\alpha$ , PGE2 and nitrite) more strongly in cells challenged with INF- $\gamma$  than LPS, and silencing HO-1 or Nrf2 with shRNA differentially affected the levels of inflammatory markers. These findings indicate that some small activators of Nrf2/HO-1 are effective modulators of microglia inflammation and highlight the chemical scaffolds that can serve for the synthesis of potent new derivatives to counteract neuroinflammation and neurodegeneration.

**Keywords:** BV2 microglia; Nrf2; HO-1; small molecule activators; heme metabolism; inflammation.

## 1. Introduction

Inflammation in the brain is considered to be a significant cause underlying the development of neurodegenerative disorders [1]. Microglia, the resident macrophages of the brain, reacts to systemic and local inflammation as well as other stressful stimuli by activating a

stress response. This response involves additional production of inflammatory molecules, reactive oxygen species and other mediators that may lead to detrimental effects. However, microglia activation is also important for stimulation of repair mechanisms and protection of neurons, indicating the dual role covered by microglial cells and emphasizing the delicate balance existing between pro- and anti-inflammatory responses in the context of brain pathophysiology.

In common with other cell types, microglia expresses the Nrf2 and heme oxygenase-1 (HO-1) systems, which are essential for counteracting oxidative stress and inflammation [2,3]. Nrf2-deficient mice are more susceptible to oxidative stress [4] and inflammatory disorders including systemic inflammation [5], localized inflammation [6] and neuroinflammation [7]. Nrf2 works up-stream of HO-1, being a transcription factor sensitive to redox changes that lead to up-regulation of several cytoprotective and detoxifying enzymes. Under normal conditions, Nrf2 is retained in the cytoplasm in a 'silent' form by its repressor protein, Keap-1, which contains a subset of 27 highly reactive cysteine residues. Oxidative and environmental stimuli modify the cysteine residues of Keap-1, thus enabling translocation of Nrf2 to the nucleus where it binds to the antioxidant responsive element (ARE) located in the promoter region of detoxifying genes, including HO-1 [8]. Although Nrf2 and HO-1 are clearly linked in eliciting a protective action, the expression of HO-1 can also be modulated by others transcriptional regulators depending on the stimulus. In addition, HO-1 *per se* exerts a catalytic function by degrading heme to generate carbon monoxide (CO), iron and bilirubin as final by-products. These products account for many of the beneficial properties of HO-1 [9,10], suggesting that pharmacological manipulation of the Nrf2/HO-1 axis could have important implications for limiting neuroinflammation. This notion is also supported by data obtained with CO-releasing molecules (CO-RMs), specific carriers of CO in biological systems that reduce the production of inflammatory markers in macrophages and microglia cells in culture [11,12] and can decrease brain damage and inflammation in a rat model of hemorrhagic stroke [13].

Our interest in Nrf2/HO-1 activators was initially motivated by results showing that curcumin, a natural polyphenolic compound, increased HO-1 levels in endothelial cells and elicited protection against H<sub>2</sub>O<sub>2</sub>-mediated oxidative damage in a HO-1-dependent manner [14].

We subsequently reported that HO-1 induction by curcumin was modulated by Nrf2 [15] and we studied other naturally-derived substances, such as chalcones and caffeic acid phenethyl ester, as promising inducers of the HO-1 pathway [16]. We also showed that the Michael acceptor functionality common to the chemical structure of many of these inducers is pivotal for promoting HO-1 up-regulation [17]. Because of their reliable response to inflammatory challenges, microglial cells in culture represent a good model to assess (brain) cellular adaptations following exposure to Nrf2/HO-1 activators.

In this study we performed an extensive literature search and selected 56 different compounds that were compared and evaluated for their ability to induce HO-1 and modulate inflammation in BV2 microglial cells. Using shRNA technology, we assessed the contribution of HO-1 and Nrf2 in mediating anti-inflammatory actions. We further concentrated on heme metabolism, showing that some compounds stimulate bilirubin production while others do not, despite their shared ability to up-regulate HO-1 expression. Our findings provide new insights on the chemical features and biochemical properties of small activators of Nrf2/HO-1 that are relevant for the control of inflammatory processes in microglia cells.

## **2. Materials and Methods**

### *2.1. Chemicals and reagents*

Supercurcumin (containing 95% curcuminoids and piperine) was purchased from Life Extension, sildenafil from Sinova Inc (Bethesda, USA), 2,2'-dihydroxychalcone from Indofine Chemical Company Inc (Hillsborough, USA), hemin and tin protoporphyrin IX (SnPPIX) from Frontier Scientific (Logan, USA) and mouse interferon- $\gamma$  (INF- $\gamma$ ) from ThermoFisher Scientific (Illkirch Cedex, France). All other chemicals and reagents were from Sigma unless otherwise stated.

### *2.2. Cell culture*

BV2 mouse microglial cells were kindly donated by Professor Rosario Donato (University of Perugia) and Dr Adjanie Patabendige (Institute of Infection & Global Health, Liverpool). Cells were cultured in either 75 cm<sup>2</sup> flasks, 6-well or 24-well dishes containing RPMI medium

supplemented with 10% FBS, 4mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and grown in a 5% CO<sub>2</sub> atmosphere at 37°C.

### *2.3. Search for Nrf2/HO-1 activators*

A literature search for compounds that induce HO-1 or Nrf2 was carried out using PubMed. The keywords used for the search were: a) HO-1 inducers and, b) Nrf2 inducers. The CAS databases were also searched using SciFinder to identify the chemical structure of various HO-1 inducers/Nrf2 activators reported in the literature. The SciFinder search used the same keywords as the PubMed search. From an original list of over 100 compounds identified in our preliminary search we created a compound library of 56 chemicals to be tested (Table 1); as some of the compounds were natural extracts or synthesized by the authors, the criteria for this selection were based on commercial availability of the substances and whether their chemical structure allowed for feasible structural modification.

### *2.4. Immunoassay for HO-1 protein expression*

A medium-throughput screening assay was utilized to determine the effect of the 56 compounds on HO-1 protein expression in BV2 cells. Confluent cells grown in 6-well dishes were treated for 6 or 24 h with increasing concentrations (5, 10 or 20 µM) of each compound using also an internal positive control (10 µM hemin) for HO-1 protein expression in each experiment. DMSO, the solvent of most of the compounds assessed (see Table 1 for solubility information), was used in control cells. At the end of the incubation, cells were scraped and collected in PBS at 4 °C. Samples were analyzed for HO-1 protein levels according to the protocol for the mouse ELISA (HO-1 (mouse) Immunoset, Vinci-Biochem).

### *2.5. Cytotoxicity*

All 56 compounds were tested to evaluate their possible cytotoxic effects. BV2 cells were grown to confluence in 24-well dishes and exposed to 5, 10 or 20 µM of each compound for 24 h. Cells were also treated with a 1% Triton solution prepared in medium as a positive control (100% cytotoxicity). Cell viability was determined using a lactate dehydrogenase (LDH) release assay

(Cytotoxicity detection kit (LDH), Roche) according to manufacturers' instructions. Briefly, at the end of the incubation supernatants were collected and centrifuged at 250 x g to remove any residual cell debris. The reaction mixture was added to the cell-free supernatant, and colour development was measured spectrophotometrically at 500 nm.

#### *2.6. Heme oxygenase activity assay*

Cells cultured in 100 mm diameter petri dishes were collected 6 h after incubation with selected inducers (20  $\mu$ M) and assessed for heme oxygenase activity. The assay is based on the spectrophotometric determination of bilirubin as the final product of heme degradation by heme oxygenase [18]. Cell samples were incubated with the substrate hemin, NADPH, liver cytosol (a source of biliverdin reductase) and other co-factors to sustain heme oxygenase activity. The reaction was allowed to proceed for 1 h at 37 °C in the dark and was terminated by addition of chloroform to extract the bilirubin produced. Bilirubin was measured spectrophotometrically as described before and calculated in picomoles bilirubin/mg protein/60 min [19].

#### *2.7. Determination of bilirubin released in the culture medium*

Confluent cells cultured in 60 mm diameter petri dishes were used for this assay. Cells were incubated with HO-1 inducers/Nrf2 activators for 6 or 24 h in the presence or absence of 10  $\mu$ M hemin in a final volume of 1 ml. At the end of the incubation, 0.5 ml of culture supernatant was added to 250 mg of BaCl<sub>2</sub> and vortex-mixed thoroughly. Benzene was added to the mixture and tubes were vigorously vortex-mixed again. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation and bilirubin was measured spectrophotometrically [19,20]. Bilirubin was calculated in picomoles/ml of medium.

#### *2.8. Real-time PCR assay*

Confluent BV2 cells cultured in 6-well dishes were exposed for 4 h to certain compounds at 10  $\mu$ M concentration. This time point was chosen based on preliminary experiments showing that maximal mRNA levels were achieved 4 h after incubation of cells with 10  $\mu$ M carnosol (data not shown). Total RNA was extracted from BV2 cells using the Qiagen RNeasy Mini Kit (Qiagen) and

then reverse-transcribed using the Superscript First-Strand Synthesis System (Invitrogen) and Oligo(dT)20. Quantitative RT-PCR reactions were performed in triplicate on a 7900 real-time PCR detection system (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix (Invitrogen). The following mouse primers were used in the reaction: HMOX1 forward primer: 5'-CCTCACTGGCAGGAAATCATC-3', HMOX1 reverse primer: 5'-CTCGTGGAGACGCTTTACATA-3', GAPDH forward primer: AAGAGAGGCCCTATCCCAAC, GAPDH reverse primer: GCAGCGAACTTTATTGATGG. PCR conditions were 50°C for 2 min, 95°C for 2 min, 45 cycles at 95°C for 15 s, and 60°C for 45 s, using GAPDH as the reference gene.

### **2.9. Measurement of cellular heme content**

Heme content was determined in cells treated with HO-1 inducers in the presence or absence of succinylacetone (1 mM), an inhibitor of heme biosynthesis and in medium containing 10 or 1% FBS. At the end of the incubation cells were washed twice with warm PBS, followed by addition of 1 ml of formic acid to solubilize the cell layer as previously described [20]. The heme concentration in the formic acid solution was measured spectrophotometrically at 398 nm ( $\epsilon = 1.56 \times 10^5$  M/cm). Heme uptake was expressed as picomole per well.

### **2.10. Western blot analysis**

Expression of biliverdin reductase (BVR), delta-aminolevulinatase synthase 1 (ALAS 1), HO-1 and Nrf2 were determined by western blot at different times after incubation with selected substances. In brief, an equal amount of proteins (20  $\mu$ g) for each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes; the nonspecific binding of antibodies then was blocked with 5% nonfat dried milk and 0.1% Tween in TBS. Membranes were then probed overnight with a rabbit polyclonal antibodies against HO-1 or biliverdin reductase (Stressgen, 1:1000 dilution), Nrf2 (Santa Cruz Biotechnology, Inc., 1:1000 dilution) and ALAS 1 (ABCAM, 1:1000 dilution). After incubation with horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology, 1:5000 dilution), bands were visualized using Pierce ECL revelation kit.



### *2.11. Silencing of HO-1 or Nrf2 using shRNA*

Small hairpin RNA specific for mouse HO-1 or Nrf2 and a negative control (sc-108080) obtained from Santa Cruz (Santa Cruz Biotechnology) were used to infect BV2 cells. Lentiviral particles were added to the cell culture in a 6-well dish and cells were incubated overnight. The following day the medium was replaced with medium containing puromycin for selection of stably transfected colonies following manufactures' instructions.

### *2.12. Nitrite assay and detection of TNF $\alpha$ and PGE2 after inflammatory challenges*

BV2 cells grown in 24-well dishes were treated for 24 h with lipopolysaccharide (LPS, 0.5  $\mu$ g/ml) or INF- $\gamma$  (15 ng/ml) in the presence or absence of test compounds (5, 10 or 20  $\mu$ M). In some experiments the heme oxygenase activity inhibitor SnPPiX (10 $\mu$ M) or bilirubin (0.5 to 10  $\mu$ M) were included. Cells in which HO-1 or Nrf2 were silenced by shRNA were also used. At the end of the incubation the supernatant was collected for measurements of nitrite levels using the Griess method [11] while TNF $\alpha$  and PGE2 levels were assessed by ELISA following manufacturers' instructions (Enzo Life Sciences, Villeurbanne, France).

### *2.13. Data analysis*

Statistical analysis was performed using Graph Pad Prism. One-way ANOVA combined with the Bonferroni or Tukey's multiple comparison tests and the T-test were applied. Differences were considered to be significant at  $P < 0.05$ . The EC50 was calculated for positive compounds used at 5, 10 and 20  $\mu$ M, thus representing an approximate rather than a definite value since a full dose response curve to each of the compound used was not performed in the present study.

## **3. Results**

### *3.1. Screening of 56 small molecules activators for HO-1 expression and cytotoxicity*

The initial literature search identified more than 100 compounds, including in some cases derivatives, which induce or activate HO-1 and/or Nrf2. These compounds had been studied by various authors using different cell types, different cell culture conditions and different experimental protocols. Thus, one aim of our study was to evaluate and standardize the effect

of these small molecules within the same cellular system and under the same experimental conditions. Some of the compounds were synthesized by the authors of the article, others were commercially available (e.g., oleanolic acid derivatives, carnosol, epigallocatechin, lansoprazole, flunarizine), while many others, such as isoegomaketone, sauchinone, or sulfuretin, were extracted from natural sources. From this initial list, 56 priority compounds were chosen to be tested for HO-1 expression after 6 and 24 h incubation at three different concentrations (5, 10 and 20  $\mu\text{M}$ ) in microglia cells. Their effect on cell viability was also assessed. We did not test concentrations higher than 20  $\mu\text{M}$  since we observed in previous experiments that some HO-1 inducers start to exhibit significant cytotoxicity around 25-30  $\mu\text{M}$  [14]. In addition, the usefulness of compounds which can increase HO-1 at concentrations higher than 20  $\mu\text{M}$  might be limited when there exists already molecules that are effective in the lower micromolar range. This is especially true if these compounds exhibit other beneficial properties, such as anti-inflammatory action, that are manifested at low micromolar doses. We note that the bioavailability of some naturally-derived compounds is limited and therefore we are unable to determine if the concentrations used in our study will be achieved in the brain after oral intake. Table 1 summarizes the results of the screening, reporting the name of the compounds, their capacity to induce HO-1 at 6 and 24 h, their solubility as well as their cytotoxic profile. From these initial experiments it is evident that many of the compounds (35) did not increase HO-1 in BV2 at the concentrations tested. These included, among others, acetaminophen, capsaicin, eriodictyol, probucol and sildenafil, which have been shown to augment HO-1 in other cell types [21-25]. We also observed that 10 compounds caused, in parallel with HO-1 induction, a release of LDH  $\geq 15\%$ , underlying some intrinsic cytotoxic properties. We set 15% LDH release as a reasonable limit for cell toxicity since the HO-1-inducing capacity of compounds should be balanced against their negative effects on cells. It is important to note that 7 out of the 10 more cytotoxic substances maintained up-regulation of HO-1 up to 24 h, suggesting that HO-1 expression is prolonged to counteract cell damage. In contrast, compounds such as carnosol (CA), dimethyl fumarate (DMF), supercurcumin (SC) and cobalt protoporphyrin IX (CoPP) stimulated HO-1 at 6 and 24 h with minor effects on cell viability (see also Table 2). Altogether, the data from this initial screening indicate that out of 56 compounds, the natural derivatives

carnosic acid (CAA), CA, DMF and SC together with the porphyrin CoPP exhibit the best HO-1 inducing/cytotoxicity profile in BV2 cells. We selected from the first set of results 10 positive leads (including hemin as our internal positive, see Table 2) and 5 negatives. Apart from their inability to promote HO-1 induction, the negatives eriodictyol (E), probucol (PRO), resveratrol (R) and sildenafil (SI) did not affect cell viability (Table 3). The only exception was plumbagin (PLU), which was highly toxic even at 5  $\mu$ M. Quantitative real time PCR confirmed induction of the HMOX1 gene for H, DMF, CA, SC (Fig. 2C) after 4 h exposure. The negatives PRO and RES also slightly increased HMOX1 gene although this effect did not translate into a significant increase in HO-1 protein expression (measured at 6 h after exposure). Indeed, no significant correlation was found between increases in mRNA and protein levels after incubation with 10  $\mu$ M H, DMF, CA, SC, PRO and RES ( $r^2 = 0.16$ , P value= 0.32), suggesting that augmented HO-1 expression by these compounds is not driven exclusively by an increase in mRNA.

### *3.2. Endogenous production of bilirubin by lead compounds*

Since many of the protective actions mediated by HO-1 are dependent on its final degradation products bilirubin and CO (and ferritin as a scavenging protein for iron), we wondered whether increased HO-1 expression by positive compounds was associated with a rise in HO-1-derived products. We measured bilirubin, which accumulates in the cell supernatant and can be detected spectrophotometrically. A rise in bilirubin also indicates a concomitant production of CO. Fig. 1A shows that of the 10 positives tested at 20  $\mu$ M, CoPP, CA, SC, CAA and *tert*-butylhydroquinone (tBH) stimulated an increase in cellular bilirubin production at 6 h and/or 24 h. The internal positive control hemin, the putative inducer and substrate of heme oxygenase activity, was used at 10  $\mu$ M concentration and promoted the expected accumulation of bilirubin [20] in the cell culture media. These results differentiate HO-1 inducers between those capable of stimulating consumption of heme by heme oxygenase and those which do not, despite their shared ability to induce high HO-1 expression. To ensure that a functional heme oxygenase was expressed following activation by the compounds, we repeated the same experiments by incubating cells with the selected substances in the presence of 10  $\mu$ M hemin. Thus, when hemin was provided exogenously as the substrate for heme oxygenase activity, bilirubin

increased both at 6 (Fig. 1B) and 24 h (Fig. 1C) with all substances examined. In the case of CoPP, CAA, 2,2-dihydroxychalcone (DHC) and tBH this increase was significantly higher than with either hemin or the substance alone, suggesting an additive or synergistic action.

### *3.3. What is the source of heme utilized by heme oxygenase following induction by certain inducers?*

The data reported above describe that certain inducers concomitantly stimulated HO-1 expression and bilirubin production in BV2 while others elicited only HO-1 induction. To better understand this phenomenon we concentrated on SC and CA and first examined if inhibition of heme biosynthesis by succinylacetone affected their capacity to increase bilirubin. For these experiments succinylacetone was pre-incubated for 16 h and maintained in the culture medium also during the entire exposure of cells to HO-1 inducers. In addition, we cultured cells in medium containing either 10 or 1% FBS, since this medium component actually contains detectable amounts of hemoglobin and could thus be an external source of heme for use by cells. We used 10  $\mu$ M hemin as positive control. Fig. 2A shows that bilirubin was produced in equal amounts when cells were exposed to hemin in the presence or absence of succinylacetone. This was expected, as hemin should enter the cell, up-regulate HO-1 and be degraded by the enzyme regardless and independently of heme synthesis inhibition. This effect was evident in medium containing either 10% or 1% FBS but with an important difference: the amount of bilirubin produced by cells incubated in 1% FBS medium was much higher compared with that measured in cells cultured in 10% FBS-containing medium. Concerning SC and CA, the results of the experiments carried out in 10% FBS do not clearly support that heme resulting from de novo synthesis was used as a substrate for production of bilirubin by SC or CA, although succinylacetone showed a non-significant tendency to decrease bilirubin levels with SC (Fig. 2A, left panel). On the contrary, our data point to FBS as the major source of heme for the production of bilirubin, since this increase was completely absent in BV2 cells incubated with SC or CA in 1% FBS (Fig. 2A, right panel). The measurement of cellular heme content indicated that treatment of cells with hemin, but not with SC or CA, increased heme levels and that this effect was much more pronounced in cells cultured in 1% FBS than 10% FBS (Fig. 2B).

Succinylacetone caused a slight, non significant decrease in heme content (Fig. 2B) at 1 mM; we decided against the use of this compound at higher concentrations to avoid possible cytotoxicity [26].

#### *3.4. Effect of small activators on expression of heme synthesis and degradation proteins (ALAS 1 and BVR)*

The pathways involving heme metabolism encompass multiple cytosolic and mitochondrial enzymes that regulate various passages of heme synthesis and degradation. The fact that some HO-1 inducers stimulate bilirubin production, while others do not, was intriguing and prompted us to explore whether pathways working alongside HO-1 in the heme metabolic reactions were also affected by the inducers. To confirm the results obtained with succinylacetone, we verified the expression levels of ALAS 1, the rate-limiting enzyme in heme biosynthesis [27] following 6 h incubation with selected substances. As shown in Fig. 3A, the expression of ALAS 1 tended to decrease in BV2 cells exposed to the positives SC, CA and DMF and the 2 negatives PRO and R but this difference was not statistically different (P value= 0.1352). In addition, we aimed to determine whether inducers changed the levels of BVR in parallel to HO-1. BVR is an inducible protein that reduces biliverdin to bilirubin but also exhibits cytoprotective and anti-inflammatory properties independently of HO-1 [28]. However, BVR expression levels remained virtually unchanged after 6 h exposure to SC, CA, DMF, PRO and R (Fig 3 B). We also tested if N-acetylcysteine (NAC, 1 mM), an antioxidant and a precursor of glutathione synthesis, influenced HO-1 induction by the inducers. Fig 3C shows that pre-incubation of BV2 with NAC diminished the increased heme oxygenase activity elicited by all the inducers evaluated (including hemin), suggesting a contribution of thiol/redox reactions in the mechanisms underlying HO-1 induction by Nrf2/HO-1 small molecule activators.

#### *3.5. Small molecules differentially modulate inflammatory markers in BV2 cells challenged with LPS or INF- $\gamma$*

The ability of the selected compounds to modulate LPS or INF $\gamma$ -induced inflammation in BV2 cells was assessed. We were keen to distinguish the response to LPS, which directly activates

TLR4 receptors and stimulates acute inflammation, from that of INF- $\gamma$ , a cytokine derived from peripheral immune cells that may better mimic a low level of chronic inflammation. Nitrite, an index of nitric oxide production, was measured in the cell supernatant 24 h after co-incubation of small activators with INF- $\gamma$  or LPS. This protocol differs from our previously published works [29], in which we pre-incubated inflammatory cells with HO-1 inducers at least 6 h prior to LPS challenge, thus ensuring high expression of HO-1 before the application of the inflammatory stimulus. Fig. 4 shows that most positives used at 20  $\mu$ M concentration decrease nitrite in cells challenged with INF- $\gamma$ ; tBH, CA, CoPP, ISL, SC and S were all effective at the highest concentration while CA, CoPP and S elicited marked decreases also at 5 and 10  $\mu$ M. Inhibiting heme oxygenase activity with SnPPIX partially reversed the reduction in nitrite caused by tBH, CA, DMF, S and SC (Supplementary Fig. S1). We also measured INF- $\gamma$ -induced nitrite levels in the presence of the negative compounds since they may exert anti-inflammatory properties independent of HO-1. In these experiments we observed that E and PLU markedly diminished nitrite production, although this effect was probably due to high cytotoxicity in the case of PLU (Fig. 5). When inflammation was triggered by LPS we observed a very good concentration-dependent inhibition of nitrite production by all positives (Fig. 6), with CA, CoPP, S and SC being the most potent. However, SnPPIX did not seem very effective in reversing this effect with all the compounds tested (Supplementary Fig. S2). As observed with INF- $\gamma$ , some negatives, such as E, R and PLU could also decrease nitrite production stimulated by LPS (Fig. 7). We found that the combination of sulphoraphane with LPS or INF- $\gamma$  caused increased microglia toxicity *in vitro* (data not shown). These results confirm that HO-1 inducers can attenuate inflammatory responses in microglia cells and suggest that the contribution of the HO-1 pathway to this effect is stronger for INF- $\gamma$ -stimulated inflammation than for LPS. In addition, these data highlight that some negatives still exert anti-inflammatory action which likely involves modulation of HO-1-independent mechanisms.

### 3.6. Correlation between bilirubin production induced by small activators and nitrite levels

As already described, HO-1-derived products mediate many of the defensive properties of heme oxygenase. Therefore, we correlated the levels of bilirubin produced by positives versus

the amount of nitrite measured after exposure of cells to INF- $\gamma$  or LPS in the presence of the compounds. We excluded compound S from this analysis since the LDH release in cells exposed to 20  $\mu$ M of the compound alone was 15.8% but markedly increased to >70% when exposed simultaneously with INF- $\gamma$  or LPS (data not shown). Thus, the decrease in nitrite in the presence of compound S is most likely a consequence of substantial cell toxicity rather than an effect mediated by HO-1 induction. The results of this correlation are very interesting as they underline an inverse, significant relationship between bilirubin and nitrite production in the case of cells challenged with INF- $\gamma$  (Fig. 8A) but a weaker and non-significant relationship for LPS-treated cells (Fig. 8B). These results suggest that HO-1 induction and its enzymatic reaction are linked to the anti-inflammatory action of the positive compounds tested. The data are also in line with those obtained with the heme oxygenase inhibitor, highlighting a stronger reliance of positives on HO-1 in counteracting inflammation caused by INF- $\gamma$  than LPS, at least concerning the nitrite response.

### *3.7. Contribution of HO-1 and Nrf2 in the modulation of inflammatory markers by small molecule activators*

To assess whether the Nrf2/HO-1 contributed to the inflammatory-modulating properties of small molecule activators, we treated cells with shRNA to knock down HO-1 or Nrf2 and examined the generation of nitrite, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced by INF- $\gamma$  or LPS. Control cells were treated with scrambled shRNA and all compounds were used at 10  $\mu$ M concentration. Fig. 9 illustrates the data obtained in INF- $\gamma$ -challenged BV2. As shown in Fig. 9A, nitrite production increased in control cells incubated with INF- $\gamma$ . Silencing of HO-1 or Nrf2 further increased nitrite levels and this effect was more pronounced in Nrf2-lacking cells. Nitrite was substantially decreased by tBH, CA and CoPP and, while down-regulation of HO-1 or Nrf2 significantly reversed this outcome in the case of tBH and CA, nitrite levels remained consistently low in scrambled, HO-1 or Nrf2 shRNA-treated cells in the presence of CoPP. The nitrite profile observed with the positive DMF or the negatives PRO and R did not differ from that of cells exposed to INF- $\gamma$  alone. Measuring PGE<sub>2</sub> provided an interesting and unexpected picture as this inflammatory mediator was increased in scrambled-

infected cells challenged with INF- $\gamma$  but was reduced to control levels in HO-1 or Nrf2-silenced cells (Fig. 9B). Levels of PGE<sub>2</sub> were similar to control in scramble, HO-1 or Nrf2 shRNA-treated cells in the presence of INF- $\gamma$  and all the positives or negatives tested. Similarly, the production of TNF- $\alpha$  was increased by INF- $\gamma$  in scrambled-infected cells (Fig. 9C) but was undetectable in HO-1 or Nrf2-silenced cells. Most of the positives and the two negatives tested decreased to some extent TNF- $\alpha$  levels stimulated by INF- $\gamma$  in scrambled control cells, however, knocking down HO-1 or Nrf2 did not restore TNF- $\alpha$  production. The only exception to this trend was observed with the positive DMF. In fact, TNF- $\alpha$  was not inhibited in scrambled control cells co-incubated with INF- $\gamma$  and DMF, was markedly reduced in HO-1 silenced cells but its levels were re-established in Nrf2-silenced BV2. These data collected in INF- $\gamma$ -challenged microglia highlight the following: 1) Nrf2/HO-1 small molecule activators modulate the INF- $\gamma$ -mediated inflammatory response; 2) HO-1 and Nrf2 appear implicated in mediating the effect of small molecule activators on nitric oxide production but not for PGE<sub>2</sub> or TNF- $\alpha$  and 3) the two negative compounds assessed exhibit independent inflammation modulating properties.

Challenge of BV2 with LPS generated a somewhat diverse outcome (Fig. 10). For example, the positives tested reduced nitrite production but to a smaller extent compared to the results observed with INF- $\gamma$  (Fig. 10A). The most effective compound in this set of experiments was CA and silencing of Nrf2, rather than HO-1, more strongly reversed this effect also in the case of the negatives PRO and R. Furthermore, and in line with the data acquired from INF- $\gamma$ -treated cells, LPS stimulated a substantial PGE<sub>2</sub> production that was decreased in Nrf2-silenced cells but even more when HO-1 was down-regulated (Fig. 10B). The presence of tBH, CA and DMF markedly diminished PGE<sub>2</sub> in scrambled, HO-1 or Nrf2-treated cells and similar trends were observed with the negatives R and to a lesser extent PRO. In contrast to the INF- $\gamma$  data, CoPP did not alter the response compared to LPS alone. The final measurement of TNF- $\alpha$  indicated that this marker increased in control scramble cells stimulated with LPS (Fig. 10C). Importantly, this increase was substantially augmented when HO-1 or Nrf2 were silenced. Apart from CoPP, the positives tBH, CA, DMF and the negative R diminished TNF- $\alpha$  induced by LPS in control cells and a tendency to reverse this effect was observed mostly in cells silenced for HO-1. Thus, we observe that following LPS stimulation small molecule activators differentially affect the



markers of inflammation we chose to examine. The results also suggest that HO-1 and/or Nrf2 contribute to the effect mediated by the compounds either by counteracting the production of certain markers (nitrite and TNF- $\alpha$ ) or by promoting their generation (PGE<sub>2</sub>). Altogether, the data also emphasize important differences between INF- $\gamma$  and LPS-induced inflammation and the roles of HO-1 and Nrf2 in the cellular response to these stimuli.

#### 4. Discussion

Manipulation of the Nrf2/HO-1 pathways has been shown experimentally to protect against a variety of conditions characterized by oxidative damage and inflammation [3,30] and targeting Nrf2/HO-1 with appropriate pharmacologically-active compounds may help to reduce the progression of chronic diseases, including neurodegeneration. It is well established that microglia activation following inflammatory challenge modulates neuroprotection [1,31], suggesting that controlling microglia activation by pharmacological approaches may be one useful step towards the reduction of neuronal damage.

We report in this study the results of a screening test of 56 compounds on the up-regulation of the cytoprotective protein HO-1 in BV2 microglia cells *in vitro*. The compounds were selected over more than 100 found during a literature search where HO-1 and/or Nrf2 were the keywords used. We compared their HO-1 stimulating capacity with their effect on cell viability. We selected 10 positives (including hemin) and 5 negatives to further examine their effect on heme metabolism and modulation of the inflammatory response. This investigation identifies carnosic acid (CAA), carnosol (CA), cobalt protoporphyrin IX (CoPP), dimethyl fumarate (DMF) and supercurcumin (SC) as the compounds eliciting the best HO-1 induction and low cytotoxicity. We also found that the compounds differentiate between those capable of promoting the generation of HO-1-derived products (CA, CoPP, SC) and others that do not, indicating that HO-1 induction by specific molecules may be independent from its heme-degradation function. When assessed for their inflammatory-modulating properties, we observed that the positives reduced more strongly inflammatory mediators stimulated by INF- $\gamma$  than LPS. In addition, the use of shRNA to silence HO-1 or Nrf2 showed that the two pathways

contribute to the effect of the compounds to counteract nitric oxide production but not PGE<sub>2</sub> or TNF- $\alpha$  in cells challenged with INF- $\gamma$ , while HO-1 and Nrf2 were substantially implicated in modulating all the inflammatory markers examined after LPS stimulation. These data highlight the pleiotropic activities of the compounds examined in this study, the different response to LPS and INF- $\gamma$ -mediated inflammation and the importance of comparing compounds with supposedly similar biological activity within the same cellular system.

The majority of the positive compounds studied in this report possess electrophilic features, which are necessary for activation of Nrf2 and the induction of Nrf2-dependent genes such as HO-1. However, despite investigating many substances, the best profile for HO-1 induction/low cell toxicity was mostly achieved by some of the original compounds first identified many years ago as HO-1 inducers (i.e. curcumin) [14] and some later identified electrophilic compounds (carnosol, chalcones) [32-34], suggesting that their chemical structure is perhaps unique and 'evolutionarily' selected to specifically activate the Nrf2/HO-1 pathway. This chemical feature is the base for the development of synthetic triterpenoids, a group of Nrf2 activators recently demonstrated to reduce neurotoxicity, oxidative stress and inflammation in a mouse model of Parkinson's disease [35]. However, the results of a recent clinical trial using a similar compound for advanced chronic kidney disease suggests that a better understanding of the biological behavior of these new molecules and a fine-tuning of their pharmacological vs. cytotoxic properties is required to fully exploit their therapeutic potential [36].

Apart from hemin, the only HO-1 inducer which is not electrophilic is CoPP. This compound can act as substrate for heme oxygenase activity, although it is less effective than the natural substrate heme [37]. However, CoPP up-regulated Nrf2 in human hepatocytes via post-transcriptional site(s) of action [38] and protected cardiac stem cells from apoptosis in a Nrf2-dependent manner [39], indicating that electrophilicity might not be the only chemical property required for Nrf2/HO-1 activation. Our data showing that NAC reduced the increase in heme oxygenase by most inducers point to oxidative stress/thiol regulation as important components for this induction. Since mouse, rat and human primary microglia have been shown to

overexpress HO-1 and activate Nrf2 following various stimuli, we suggest that the Nrf2/HO-1 activators investigated in the present study will exert similar effects also in primary cells.

Our results also show that not all HO-1 inducers stimulate bilirubin production. It was intriguing to find that for those compounds that induced HO-1 (CA and SC), the source of heme was external, i.e. the fetal bovine serum with its residual haemoglobin content (18.5 mg/100 ml according to the certificate of analysis of the FBS used in our cell culture work). This idea is supported by our data indicating that: 1) no bilirubin production was observed when cells were incubated in medium containing only 1% serums; 2) pharmacological inhibition of heme biosynthesis did not affect bilirubin release in the supernatant in the presence of CA and SC and 3) protein expression of other heme metabolism pathways directly related to heme synthesis and degradation (ALAS 1 synthase or BVR) was not changed by CA and SC. The use of exogenous heme sources was unlikely to depend from cytotoxicity since CA and SC were among the least toxic compounds at the concentrations used. A recent article by Zelenka et al. has measured bilirubin production both intracellularly and in the culture medium by HPLC, showing that bilirubin is increased in HepG2 cells following HO-1 induction by sodium arsenite and thus confirming the ability of cells in culture to generate detectable levels of bilirubin after exposure to the appropriate stimuli [40].

Our approach helped us to compare the molecules in terms of their anti-inflammatory activity, in order to select the ones that best modulate inflammation in microglia. Positive compounds, and to an extent also some negatives (E for INF- $\gamma$  and R for LPS), decreased nitric oxide generation induced by INF- $\gamma$  or LPS and inhibition of heme oxygenase activity partially reversed this effect. The degree of nitrite reduction was different for each small molecule and we noted a stronger contribution of the HO-1 pathway in cells exposed to INF- $\gamma$ - than to LPS. Indeed, the correlation analysis showed a significant inverse correlation between nitrite levels and bilirubin production elicited by the compounds in INF- $\gamma$ -treated cells. This pattern is interesting and suggests that higher bilirubin levels stimulated by some substances results in lower nitrite released by microglia following INF- $\gamma$ . However, bilirubin applied exogenously (0.5 to 10  $\mu$ M) did not change nitrite levels (data not shown). Thus, we suggest that this effect may be due to CO, produced simultaneously to bilirubin during heme degradation by heme

oxygenase, as shown previously for CO-releasing molecules in murine macrophages and BV2 cells [12,41].

In general, the inflammatory response promoted by LPS was more robust than with INF- $\gamma$ ; possibly emphasizing a different microglia response to acute (LPS) versus chronic low level inflammation (INF- $\gamma$ ). In line with this idea, Orozco *et al* have shown in a large study involving 92 mouse strains that changes in gene expression are more pronounced in primary macrophages exposed to LPS than to oxidized phospholipids, which also leads to chronic inflammation [42]. Interestingly, the study revealed that HO-1 was markedly regulated in response to oxidized phospholipids but not to LPS. Our data using cells silenced for HO-1 or Nrf2 also highlight intriguing observations. First, we noted that in the absence of positives or negatives, silencing HO-1 or Nrf2 modulated the production of inflammatory markers induced by INF- $\gamma$  or LPS; in particular, nitrite levels were enhanced while PGE<sub>2</sub> was decreased. Conversely, lack of HO-1 or Nrf2 abolished TNF- $\alpha$  release in cells challenged with INF- $\gamma$  but augmented it in the case of LPS. Thus, HO-1 and Nrf2 counteract nitric oxide production but support the generation of PGE<sub>2</sub>. This inflammatory lipid mediator deriving from cyclooxygenase-2 exhibits both pro- and anti-inflammatory properties and may have different roles depending on the condition examined. Second, knocking down HO-1 or Nrf2 influenced more nitrite production by the compounds in cells challenged with INF- $\gamma$ . The compounds (including the negatives PRO and R) completely blocked PGE<sub>2</sub> production by INF- $\gamma$  and silencing HO-1 or Nrf2 did not restore PGE<sub>2</sub> levels. This effect was similar but less pronounced in LPS-stimulated microglia. Our findings are in line with a recent report showing that R inhibited LPS-mediated stimulation of PGE<sub>2</sub> by BV2 via the mammalian target of rapamycin signalling pathway [43]. They also demonstrate that natural based molecules can exert similar anti-inflammatory activity by modulating cellular mechanisms alternative or parallel to HO-1 and Nrf2. Third, the inflammatory-regulating properties of the compounds were better differentiated in LPS-treated cells. For example, CA was the most effective regulator of the response while tBH or DMF had less influence on nitrite production but affected more PGE<sub>2</sub> and TNF- $\alpha$  levels. Although the bioavailability of these compounds may be limited *in vivo*, published

findings in animals show beneficial effects of sulforaphane, curcumin and other Nrf2 activators in brain inflammation [3,44].

In conclusion, we have identified potent activators of the Nrf2/HO-1 pathways (i.e. carnosol, cobalt protoporphyrin, dimethyl fumarate, supercurcumin) capable of modulating inflammation in BV2 microglia, suggesting a complex role of these compounds in controlling the production of inflammatory mediators. Our results also point to heme metabolism as an additional factor affecting the actions of these compounds, indicating that HO-1 induction in the absence of heme degradation may limit the cytoprotective potential of this enzyme. This screening approach suggests that modification of selected chemical scaffolds may be promising for the synthesis of new molecules to combat brain inflammation and neurodegeneration, as recently shown by Chen Q et al. with the synthesis of potent, orally active curcumin derivatives that prevented cognitive decline in a mouse model of Alzheimer's disease [45].

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**Table 1. List of compounds tested in the initial screening.**

COMPOUND	HO -1 induction 6h	HO -1 induction 24h	Cytotoxicity (≥ 15% LDH release) <sup>a</sup>	Solubility
Acetaminophen <sup>b</sup>	-	-	-	DMSO <sup>c</sup>
Acetylcarnitine	-	-	-	H <sub>2</sub> O
Allyl isothiocyanate	↑ <sup>d</sup>	↑	↑ <sup>d</sup>	DMSO
Allyl sulfide	-	-	-	H <sub>2</sub> O
Auronafin	↑	↑	↑	DMSO
Aurothioglucose hydrate	-	-	-	H <sub>2</sub> O
Aurothiomalate (sodium salt)	-	-	-	DMSO
Baicalein	-	-	-	DMSO
<i>tert</i> -Butylhydroquinone	↑	↑	↑	DMSO
Caffeic acid phenethyl ester	↑	↑	↑	DMSO
Capsaicin	-	-	-	DMSO
Carnosic acid	↑	↑ (only at 5 μM)	-	DMSO
Carnosol	↑	↑	-	DMSO
Celastrol	↑ (only at 5 μM)	↑ (only at 5 μM)	-	DMSO
Celecoxib	↑	-	-	DMSO
Chlorophyllin (sodium copper salt)	-	-	-	H <sub>2</sub> O
Cilostazol	-	-	-	DMSO
<i>trans</i> -Cinnamaldehyde	-	-	-	H <sub>2</sub> O
Cobalt protoporphyrin IX	↑	↑	-	0.01M NaOH
Costunolide	-	-	-	DMSO
Dehydrocostus lactone	↑	↑	↑	DMSO
Deltamethrin	-	-	-	DMSO
2,2-Dihydroxychalcone	↑	↑	↑	DMSO
Dimethyl fumarate	↑	↑	-	H <sub>2</sub> O
Enterolactone	-	-	-	DMSO
Epibatidine	-	-	↑	DMSO
Epigallocatechin	↑ (only at 5 μM)	-	-	H <sub>2</sub> O
Epigallocatechin gallate	-	-	-	H <sub>2</sub> O
Eriodictyol	-	-	-	DMSO
Ferulic acid	-	-	-	DMSO
Flunarazine	-	-	-	DMSO
Hemin (internal positive)	↑	not tested	not tested	0.01M NaOH
Hesperidin	-	-	-	DMSO
2-Hydroxychalcone	↑ (only at 20 μM)	-	-	DMSO
Isoliquiritigenin	↑	-	↑	DMSO
Kaempferol	-	-	-	DMSO
Lansoprazole	-	-	↑	DMSO
α-Lipoic acid	↑ (only at 5 μM)	↑ (only at 5 μM)	-	DMSO

<b>Melatonin</b>	-	-	-	DMSO
<b>Nicotine</b>	↑ (only at 5 μM)	-	-	DMSO
<b>Nordihydroguaiaretic acid</b>	↑	↑	↑	DMSO
<b>Piceatannol</b>	-	-	-	DMSO
<b>Piperine</b>	-	-	-	DMSO
<b>Plumbagin</b>	-	-	↑	DMSO
<b>Probucol</b>	-	-	-	DMSO
<b>Puerarin</b>	-	-	↑	DMSO
<b>Quercitin</b>	-	-	↑	DMSO
<b>Resveratrol</b>	-	-	-	DMSO
<b>Rottlerin</b>	-	-	-	DMSO
<b>Sildenafil</b>	-	-	-	DMSO
<b><i>trans</i>-Stilbene oxide</b>	-	-	-	DMSO
<b>Sulfasalazine</b>	-	-	-	DMSO
<b>Sulforaphane</b>	-	↑	↑	DMSO
<b>Supercurcumin</b>	↑	↑	-	DMSO
<b>Tangeretin</b>	-	-	-	DMSO
<b>Tanshinone</b>	-	-	↑	DMSO
<b>2,2,4-Trihydroxycalcone</b>	↑	-	↑	DMSO

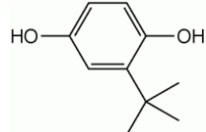
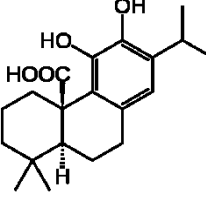
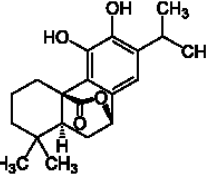
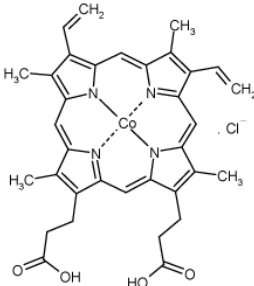
<sup>a</sup> LDH, lactate dehydrogenase release assay

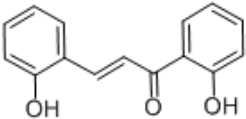
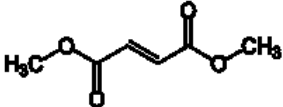
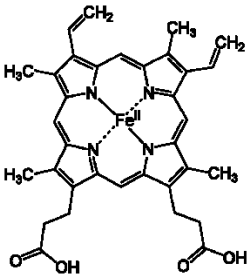
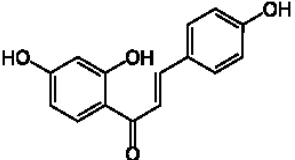
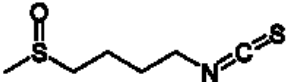
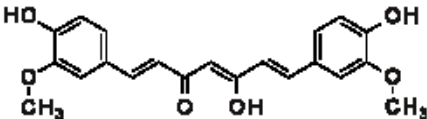
<sup>b</sup> Fifty-six compounds were selected from over 100 molecules identified through a literature search as HO-1 and/or Nrf2 inducers

<sup>c</sup> DMSO, dimethyl sulfoxide

<sup>d</sup> Arrows indicate an increased HO-1 protein expression at the time indicated or a cytotoxicity ≥ 15% when cells were exposed for 24 h to the compounds listed at 20 μM final concentration

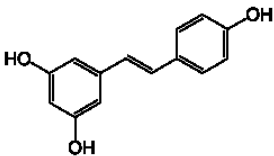
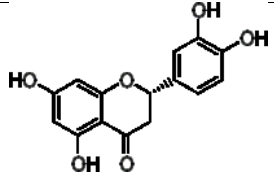
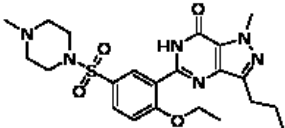
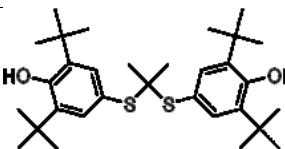
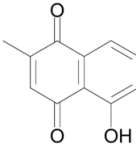
**Table 2. Chemical structure and potency of HO-1 inducers (positive compounds).**

POSITIVE COMPOUNDS	Chemical Structure	HO -1 (fold-increase)						HO-1 EC <sub>50</sub> 6h μM	Cytotoxicity % LDH release <sup>a</sup>  20 μM
		6h			24h				
		5 μM	10 μM	20 μM	5 μM	10 μM	20 μM		
<b><i>tert</i>-Butyl hydroquinone (tBH)</b>		2.5 <sup>b</sup>	5.6	7.9	2.9	3.0	4.4	9.2	94.8
<b>Carnosic acid (CAA)</b>		1.1	1.4	2.1	2.1	1.5	1.3	13.1	5.8
<b>Carnosol (CA)</b>		1.5	2.2	2.9	1.1	1.6	2.3	9.4	6.2
<b>Cobalt Protoporphyrin IX (CoPP)</b>		2.9	3.4	4.6	6.0	4.9	5.1	12.6	0

<b>2,2-Dihydroxychalcone (DHC)</b>		2.5	3.3	4.8	1.1	1.8	2.1		44.1
<b>Dimethyl fumarate (DMF)</b>		1.7	5.9	7.6	1.9	1.5	1.0	8.1	0.8
<b>Hemin (H, internal positive)</b>		n.t.	4.3	n.t.	n.t.	n.t.	n.t.	-	n.t.
<b>Isoliquiritigenin (ISL)</b>		1.0	1.3	1.7	-	-		11.8	15.5
<b>Sulforaphane (S)</b>		-	-	-	1.8	1.9	2.4	7.8*	15.8
<b>μSupercurcumin (SC)</b>		0.9	1.6	2.3	1.2	1.8	2.5	10	10.3

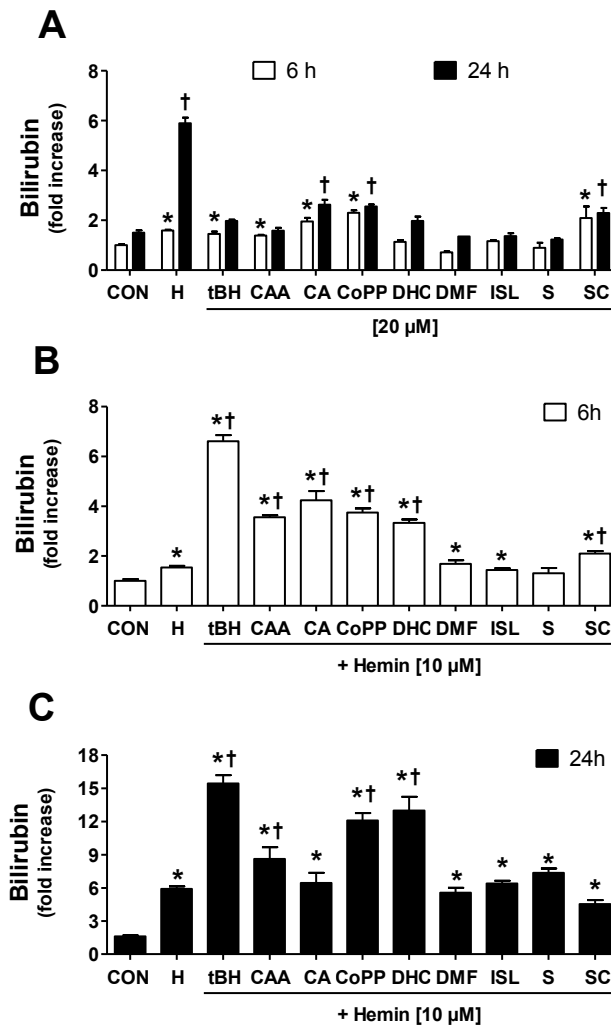
<sup>a</sup> Cytotoxicity, expressed as % of lactate dehydrogenase (LDH) release, was measured after incubation of BV2 microglia cells for 24 h with 20  $\mu$ M of the indicated compounds. <sup>b</sup> HO-1 protein expression was measured as fold-increase at 6 and 24 h after treatment of BV2 microglia cells with compounds at the concentrations indicated.

**Table 3. Chemical structure and cytotoxicity of compounds not inducing HO-1 (negative compounds).**

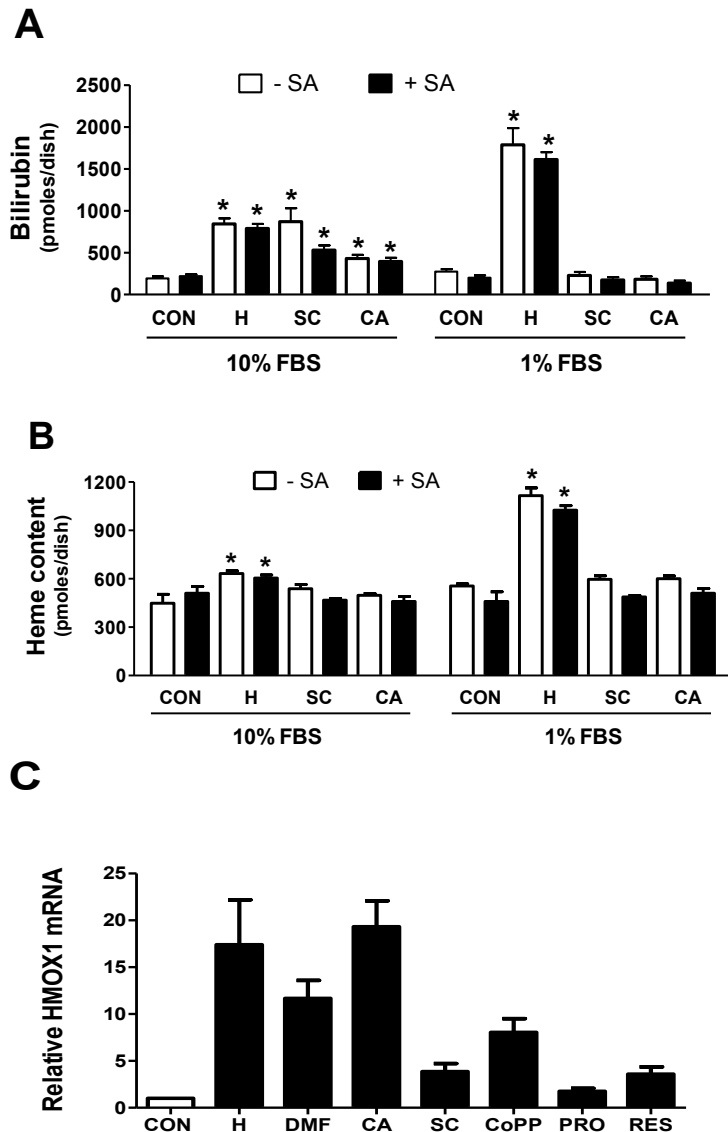
NEGATIVE COMPOUNDS	Chemical Structure	Cytotoxicity % LDH release <sup>a</sup>
Resveratrol (R)		0.2
Eriodictyol (E)		0
Sildenafil (SIL)		0
Probucol (PRO)		2.3
Plumbagin (PLU)		89.5

<sup>a</sup> Cytotoxicity, expressed as % of lactate dehydrogenase (LDH) release, was measured after incubation of BV2 microglia cells for 24 h with 20  $\mu$ M of the indicated compounds.

**FIGURE 1.** Bilirubin production following exposure of BV2 to HO-1 inducers. A) Bilirubin accumulated over time was measured in the supernatant of microglia cells 6 or 24 h after exposure to different HO-1 inducers (20  $\mu$ M). \* < *P* 0.05 vs. control 6 h, † < *P* 0.05 vs. control 24 h. B) and C) Bilirubin released in the culture medium following co-incubation of HO-1 inducers (20  $\mu$ M) and the heme oxygenase substrate hemin (H, 10  $\mu$ M) for 6 or 24 h, respectively. Bilirubin was calculated in picomoles/ml medium and is shown here as fold increase over the control. Data are the mean  $\pm$  SEM of 4-6 independent experiments per group. \* < *P* 0.05 vs. control (CON), † < *P* 0.05 vs. hemin alone (H).

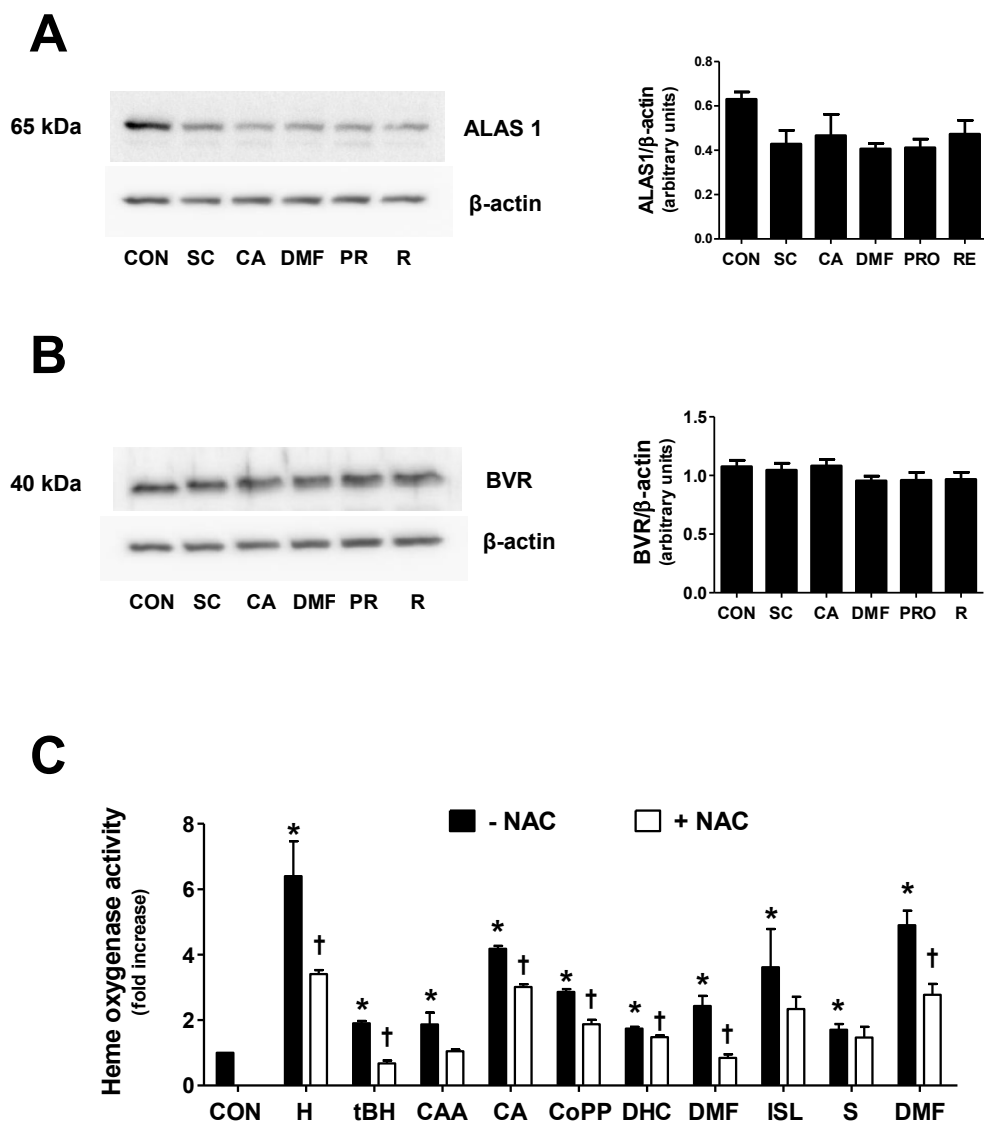


**FIGURE 2.** Cellular bilirubin production and heme levels in the presence of a heme biosynthesis inhibitor. A) Bilirubin accumulated in the culture supernatant and B), heme content measured following exposure of BV2 for 6 h to hemin (H, 10  $\mu$ M) and supercurcumin (SC, 20  $\mu$ M) or carnosol (CA, 20  $\mu$ M) in medium containing 10% or 1% FBS, in the presence or absence of succinylacetone (SA, 1 mM). Bilirubin and heme levels were assessed according to spectrophotometric assays described in Materials and Methods. C) HMOX1 mRNA levels after incubation with different inducers and negatives as measured by RT-PCR. Data are the mean  $\pm$  SEM of 3-6 independent experiments per group. \*  $P$  0.05 vs. control (CON).

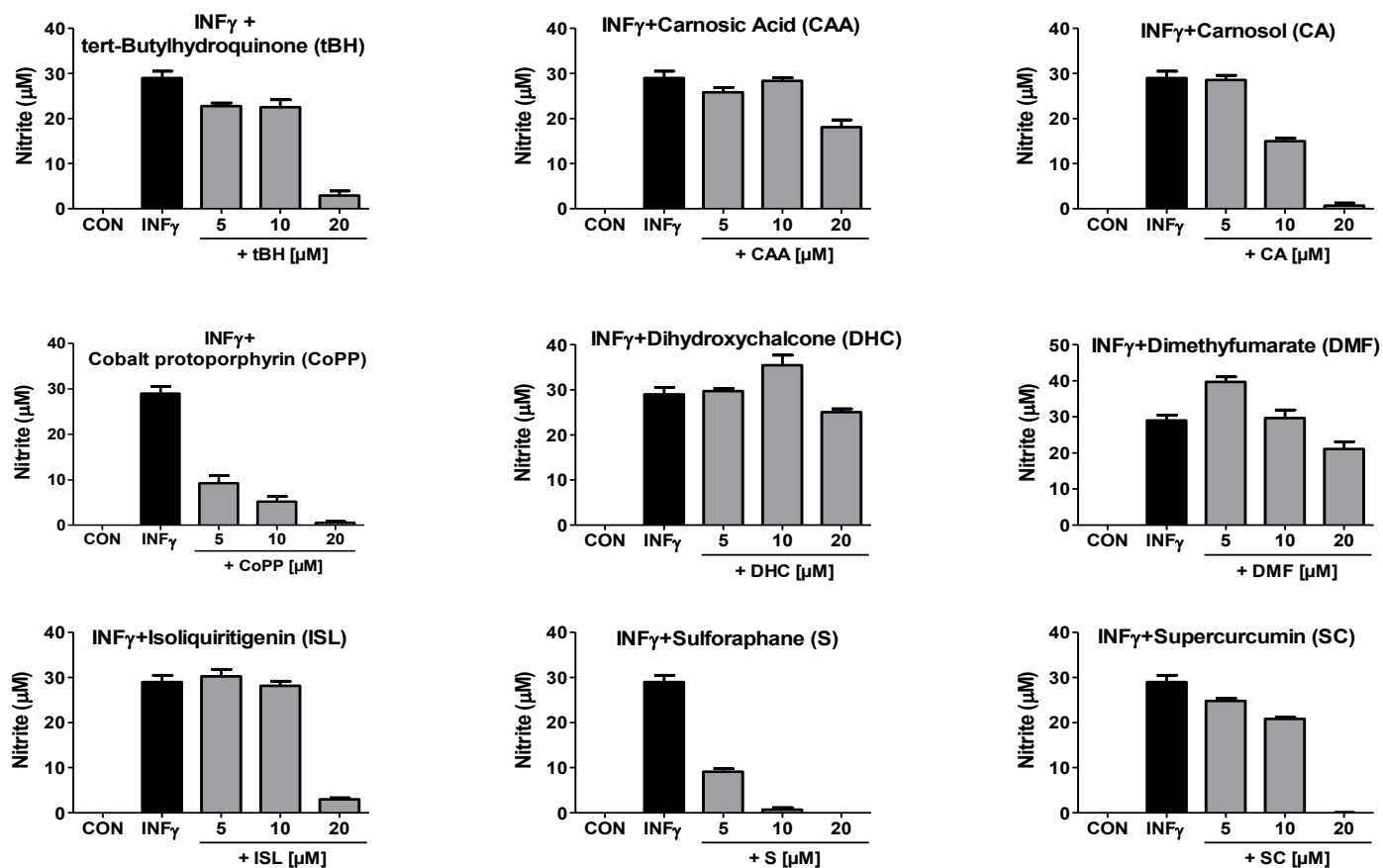




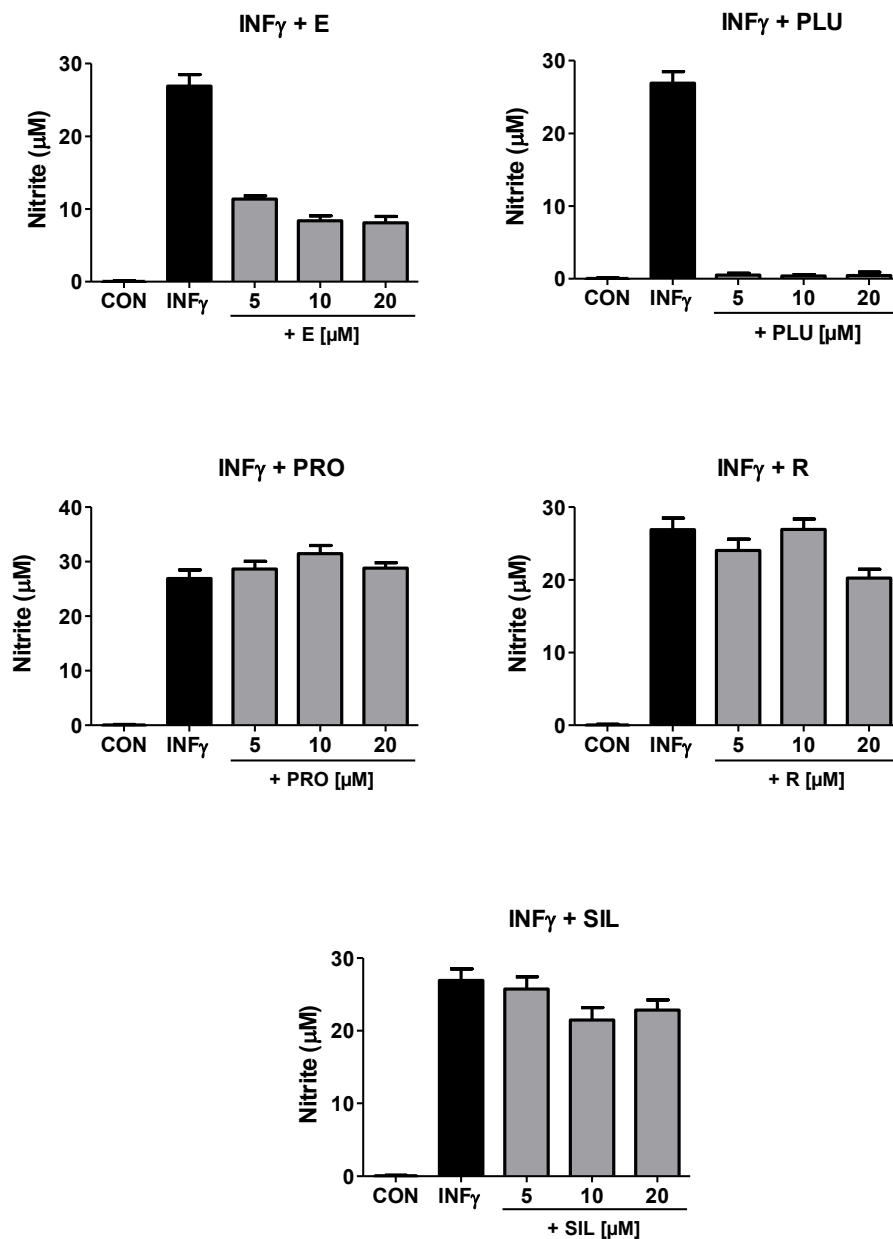
**FIGURE 3.** Small molecules activators do not increase expression of ALAS 1 or BVR. A) ALAS 1 and B), BVR expression were measured by Western Blot after 6 h incubation of BV2 cells with 20  $\mu$ M supercurcumin (SC), carnosol (CA), dimethyl fumarate (DMF), probucol (PRO) or resveratrol (R). Results represent the mean of 3 independent experiments and the corresponding densitometric analysis, normalized over the levels of  $\beta$ -actin, is depicted on the right. C) Heme oxygenase activity was determined in cells exposed to 10 different HO-1 inducers (20  $\mu$ M) in the presence or absence of N-acetylcysteine (NAC, 1 mM) using a biochemical assay well established in our laboratory. NAC partially reduced the increase in heme oxygenase stimulated by the compounds. Heme oxygenase activity was calculated in picomoles bilirubin/mg protein/60 min and expressed in fold increase over the control values. Data are the mean  $\pm$  SEM of 4 experiments per group. \* <  $P$  0.05 vs. control (CON),  $\dagger$  <  $P$  0,05 vs. HO-1 inducer alone.



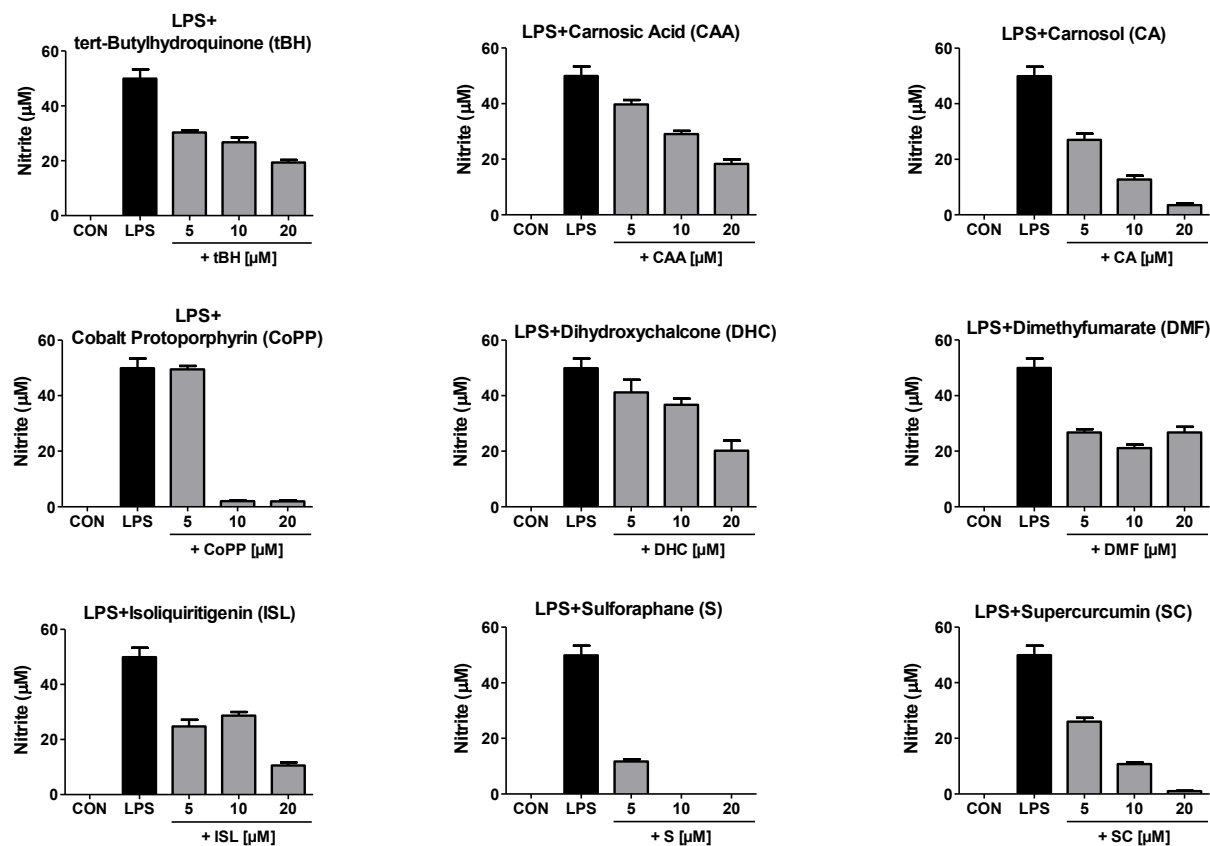
**FIGURE 4.** HO-1 inducers affect nitrite production elicited by INF- $\gamma$  in a concentration-dependent manner. Cells were exposed for 24 h to INF- $\gamma$  (15 ng/ml) in the presence of increasing concentrations of the compounds (5, 10 and 20  $\mu$ M) and nitrite levels were measured in the supernatant. All small molecule activators of Nrf2/HO-1 tended to decrease nitrite levels stimulated by INF- $\gamma$ , especially at the highest concentration tested (20  $\mu$ M). CoPP and sulforaphane (S) were the most effective even at lower concentrations. The Griess reagent was used to determine nitrite production. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.



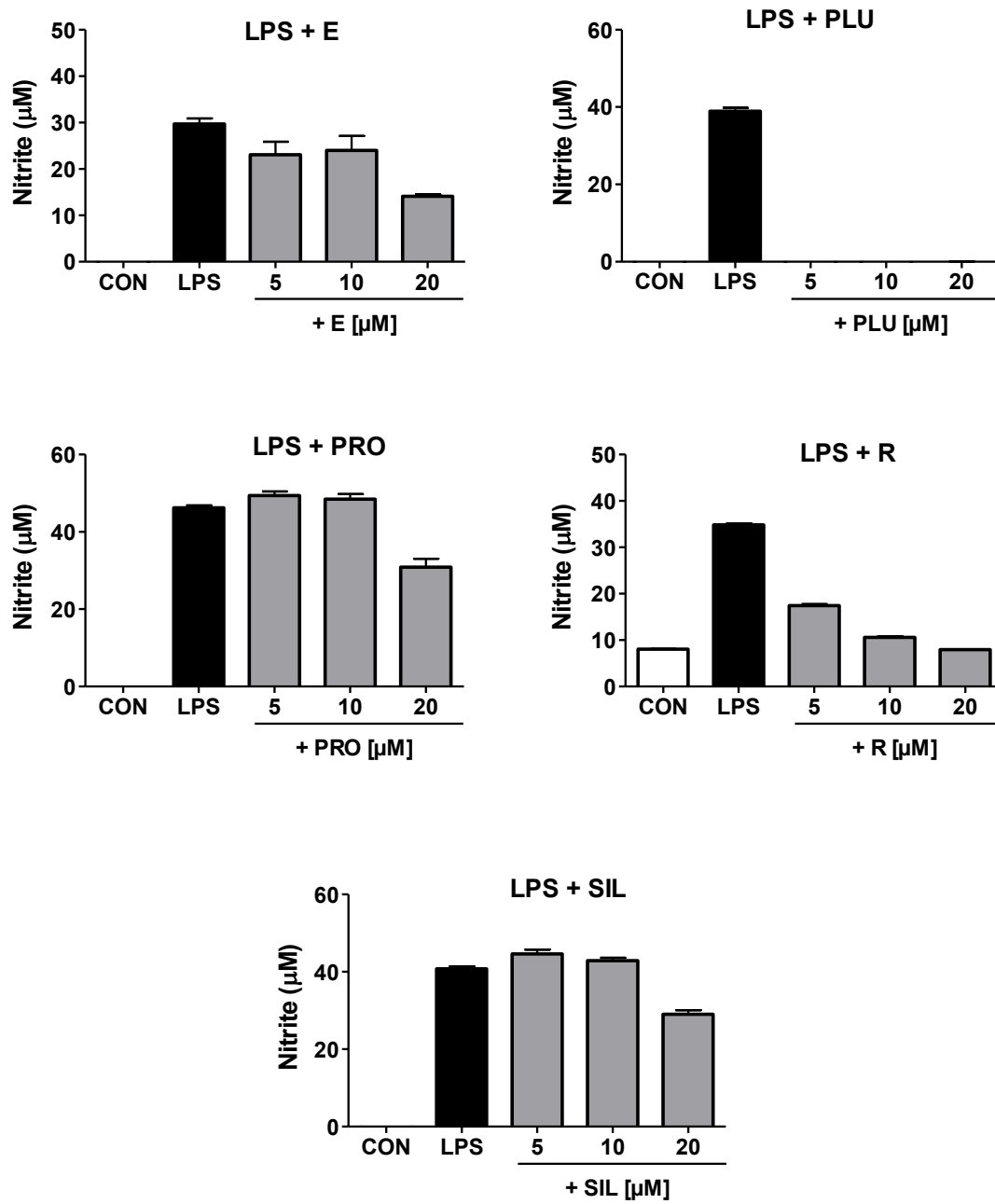
**FIGURE 5.** Effect of negatives on nitrite production elicited by INF- $\gamma$ . Cells were exposed for 24 h to INF- $\gamma$  (15 ng/ml) in the presence of increasing concentrations (5, 10 and 20  $\mu$ M) of the compounds that did not up-regulate HO-1 and nitrite levels were measured in the supernatant. Eriodictyol (E) and plumbagin (PLU) were the only ones capable of decreasing nitrite levels, although for PLU this effect is likely due to the extreme cell toxicity caused by the compound. The Griess reagent was used to determine nitrite production. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.



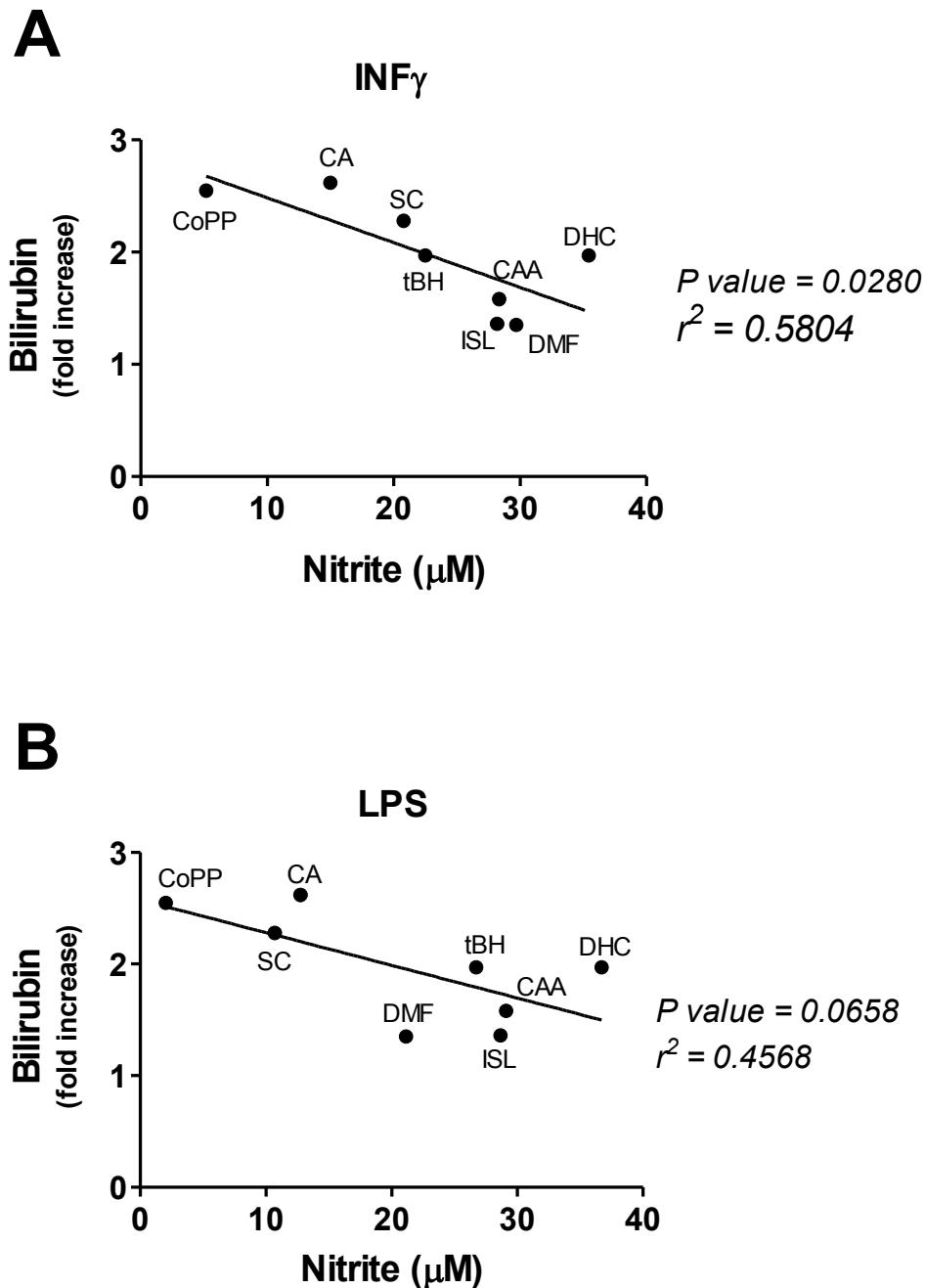
**FIGURE 6.** Small molecule activators affect nitrite production elicited by LPS in a concentration-dependent manner. Cells were exposed for 24 h to LPS (0.5  $\mu\text{g}/\text{ml}$ ) in the presence of increasing concentrations (5, 10 and 20  $\mu\text{M}$ ) of the compounds and nitrite levels were measured in the supernatant. All compounds markedly decreased nitrite levels stimulated by LPS. Carnosol (CA), CoPP, sulforaphane (S) and supercurcumin (SC) were the most effective molecules. The Griess reagent was used to determine nitrite production. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.



**FIGURE 7.** Effect of negatives on nitrite production elicited by LPS. Cells were exposed for 24 h to LPS (0.5  $\mu\text{g}/\text{ml}$ ) in the presence of increasing concentrations (5, 10 and 20  $\mu\text{M}$ ) of negatives and nitrite levels were measured in the supernatant. Eriodictyol (E) and plumbagin (PLU) were markedly decreased nitrite levels, although this effect was likely due to the extreme cell toxicity caused in the case of PLU. The Griess reagent was used to determine nitrite production. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.

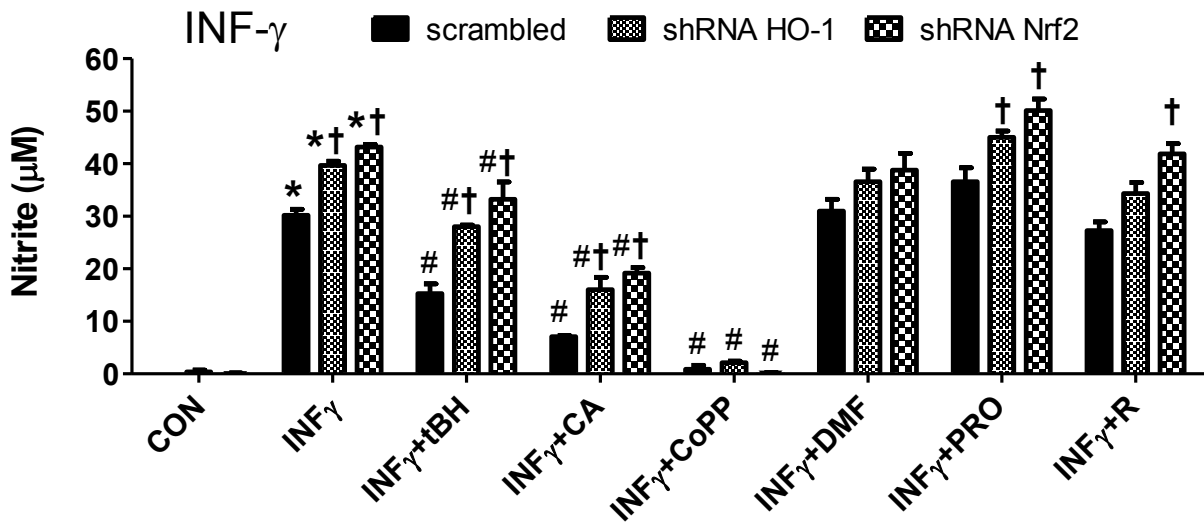


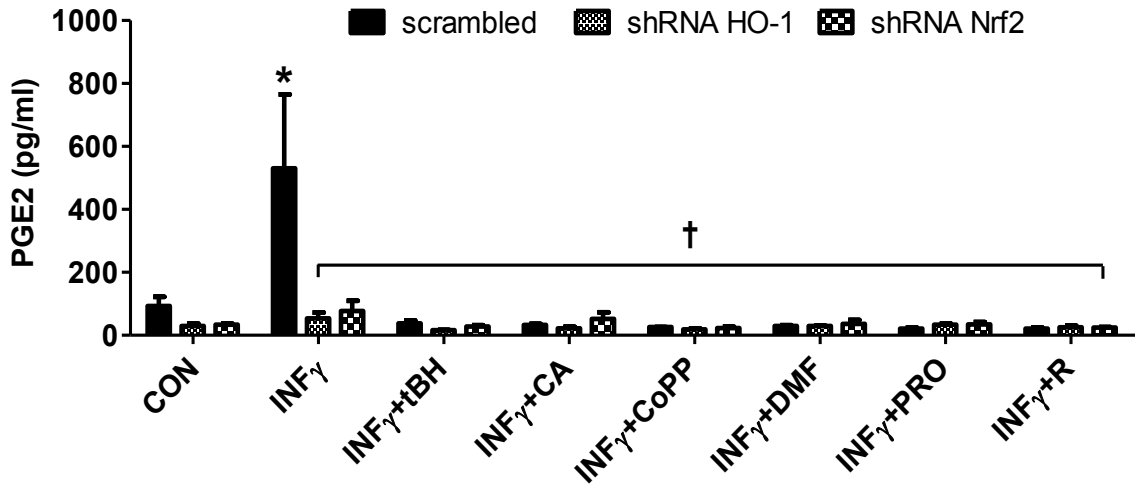
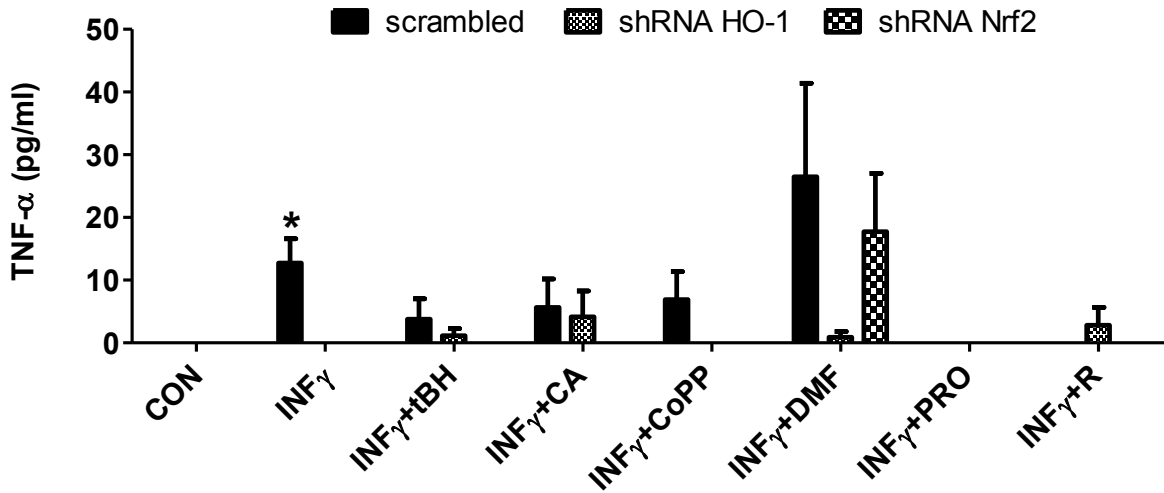
**FIGURE 8.** Bilirubin production induced by HO-1 inducers is inversely correlated with nitrite levels. A) Correlation analysis between the amount of bilirubin generated by each positive and nitrite levels following co-incubation of BV2 with INF- $\gamma$  and positive compounds. The results show that higher bilirubin production is associated with lower nitrite. B) The same analysis was performed in cells challenged with LPS, showing a similar, albeit not significant, trend.



**FIGURE 9.** Changes in the production of inflammatory mediators elicited by small molecules after INF- $\gamma$  challenge are partially controlled by the HO-1 and/or Nrf2 genes. BV2 cells were treated with scrambled control shRNA or shRNA for HO-1 or Nrf2 as described in Materials and Methods. Once transfected colonies were selected, cells were stimulated with INF- $\gamma$  (15 ng/ml) for 24 h in the presence or absence of the HO-1 inducers tert-butyl hydroquinone (tBH), carnosol (CA), CoPP and dimethyl fumarate (DMF) and the negatives probucol (PRO) and resveratrol (R) at 10  $\mu$ M concentration and the supernatant was collected for measurement of nitrite (Griess reagent) and PGE<sub>2</sub> and TNF- $\alpha$  by ELISA. A) Compounds decreased nitrite levels to different extents and silencing HO-1 or Nrf2 partially reversed this effect. B) Knock down of HO-1 or Nrf2 did not reverse the inhibition of PGE<sub>2</sub> production by compounds. C) INF- $\gamma$ -mediated TNF- $\alpha$  production was partially decreased by compounds but was not reversed in cells silenced for HO-1 or Nrf2. Data are the mean  $\pm$  SEM of 3 independent experiments per group. \*  $P$  0.05 vs. unstimulated cells (CON), <sup>†</sup>  $P$  0.05 vs. scrambled-transfected cells, #  $P$  0.05 vs. cells stimulated with INF- $\gamma$  only.

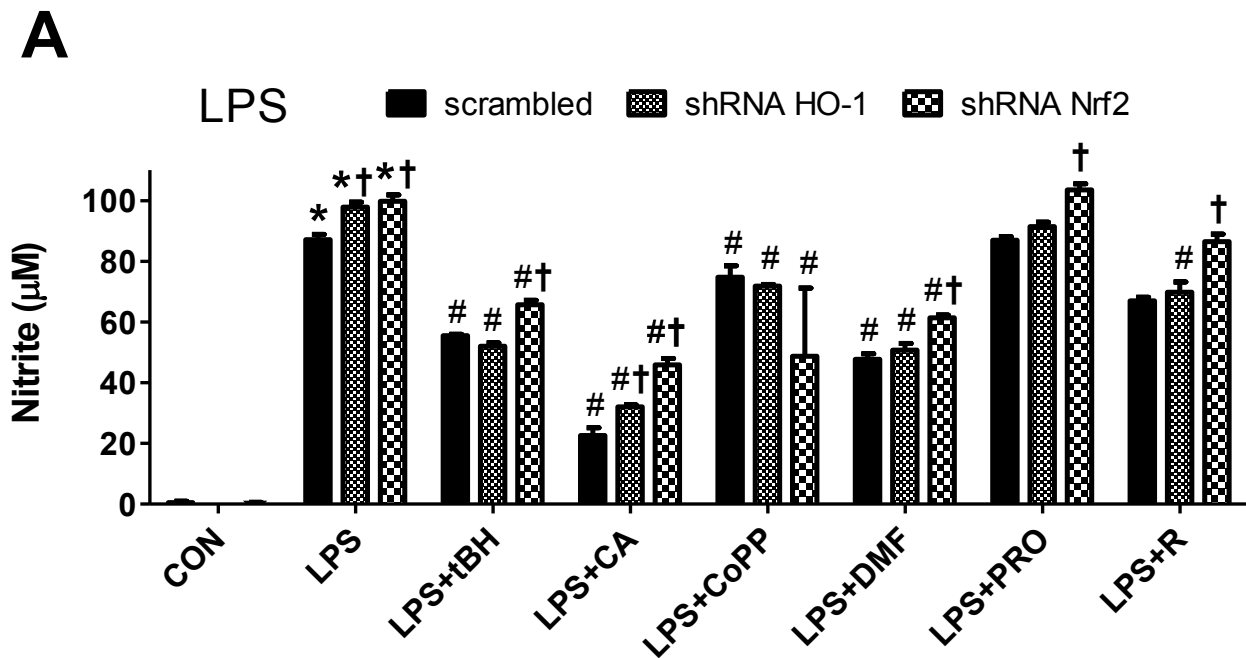
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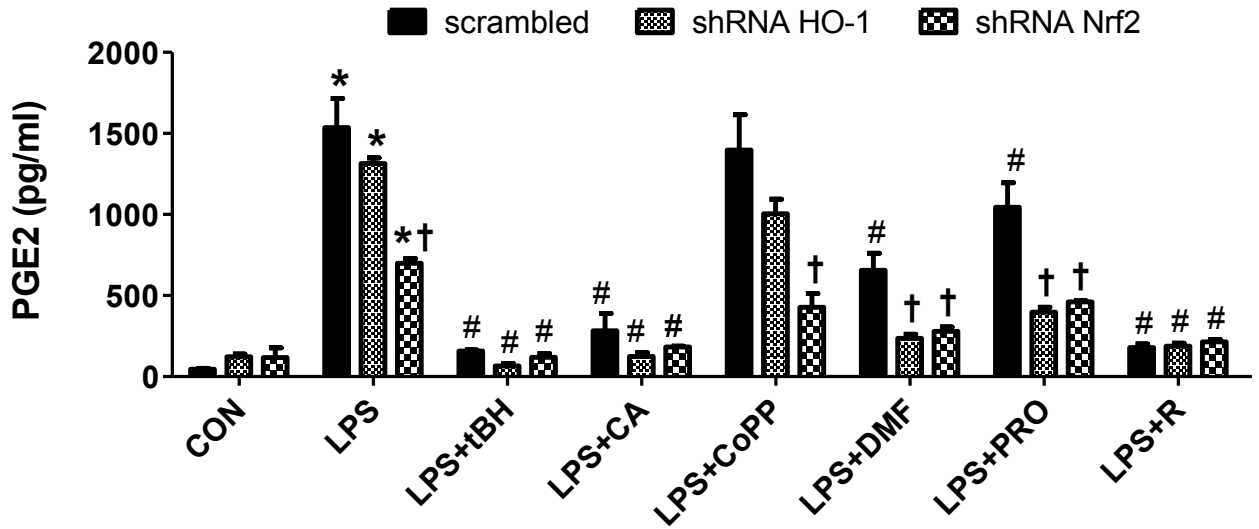
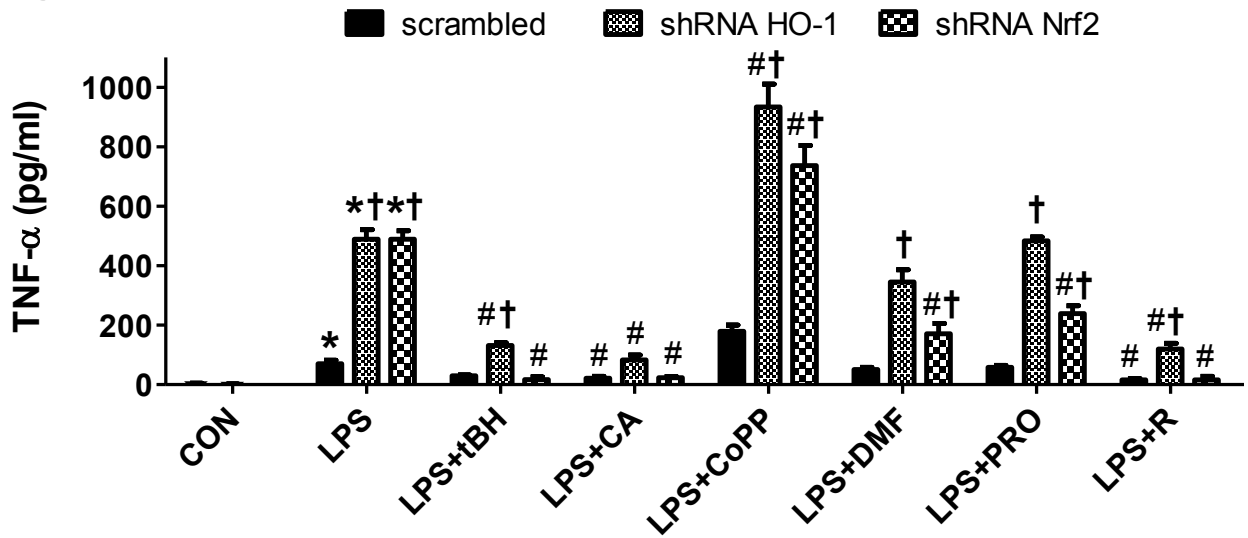


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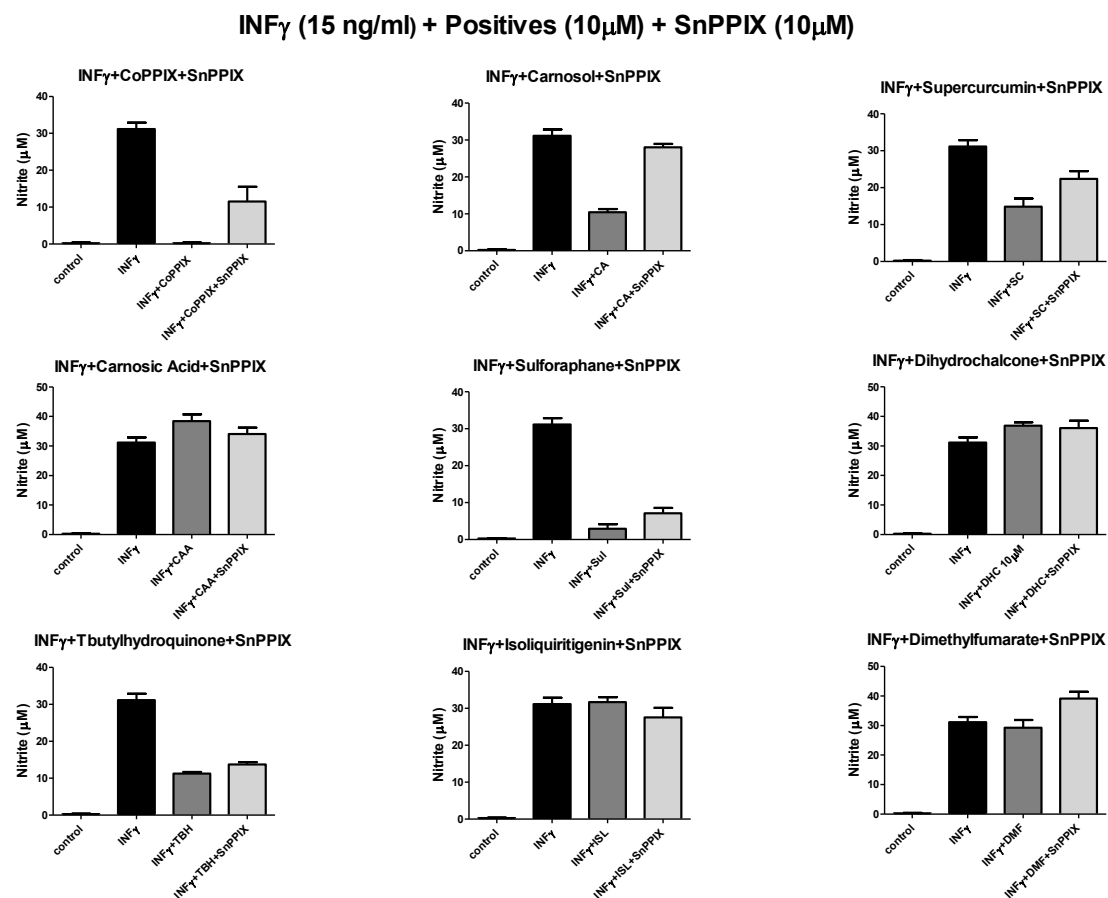


**FIGURE 10.** Changes in the production of inflammatory mediators elicited by small molecules after LPS challenge are partially controlled by the HO-1 and/or Nrf2 genes. BV2 cells were treated with scrambled control shRNA or shRNA for HO-1 or Nrf2 as described in Materials and Methods. The same treatments and measurements described for Fig. 9 were applied in cells stimulated with LPS (0.5  $\mu\text{g/ml}$ ) for 24 h. A) Compounds decreased nitrite levels to different extents and silencing Nrf2 partially reversed this effect. B) Knock down of HO-1 or Nrf2 reduced PGE<sub>2</sub> levels induced by LPS and did not reverse the inhibition of PGE<sub>2</sub> production by compounds. C) LPS-mediated TNF- $\alpha$  production was increased in cells silenced for HO-1 or Nrf2. Compounds partially decreased TNF- $\alpha$  levels and silencing HO-1 partially reversed this effect. Data are the mean  $\pm$  SEM of 3 independent experiments per group. \* < *P* 0,05 vs unstimulated cells (CON), <sup>†</sup> < *P* 0.05 vs. scrambled-transfected cells, <sup>#</sup> < *P* 0.05 vs. cells stimulated with LPS only.



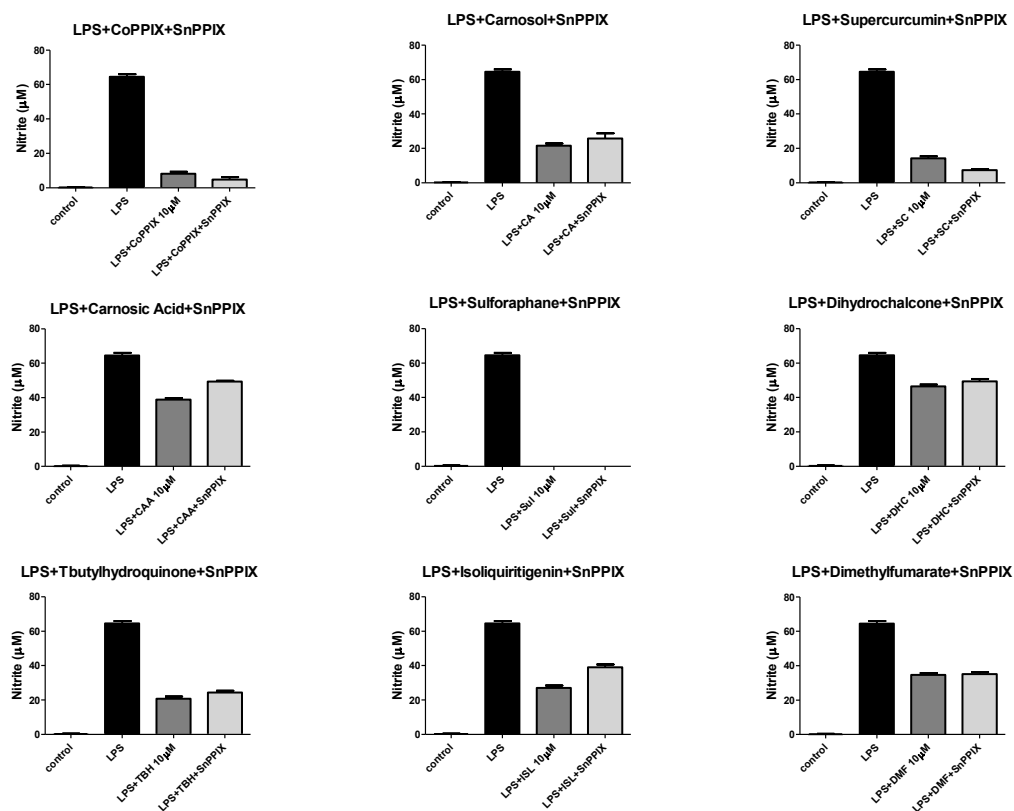
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**Supplementary Figure 1.** Inhibition of heme oxygenase activity by SnPPIX partially reverses the decrease in INF- $\gamma$ -mediated nitrite production exerted by some HO-1 inducers. SnPPIX (10  $\mu$ M), the inhibitor of heme oxygenase activity, was co-incubated with small molecule activators (10  $\mu$ M) in cells challenged with INF- $\gamma$  (15 ng/ml) for 24 h. We observed that the decrease in nitrite levels elicited by tert-butyl hydroquinone (tBH), carnosol (CA), CoPP, supercurcumin (SC) and sulforaphane (S) was partially reversed by SnPPIX. Nitrite was measured in the cell supernatant using the Griess reagent. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.



**Supplementary Figure 2.** Small contribution of the HO-1 pathway in the decrease of nitrite production exerted by certain compounds in LPS-challenged BV2 cells. SnPPIX (10  $\mu$ M) was co-incubated with small molecule activators (10  $\mu$ M) in cells challenged with LPS (0.5  $\mu$ g/ml) for 24 h. The heme oxygenase inhibitor modestly reversed the decrease in nitrite levels elicited by tert-butyl hydroquinone (tBH), carnosic acid (CAA), carnosol (CA), and isoliquiritigenin (ISL). Nitrite was measured in the cell supernatant using the Griess reagent. Data represent the mean  $\pm$  SEM of 5-6 independent experiments per group.

**LPS (0,5  $\mu$ g/ml) + POSITIVES (10  $\mu$ M) + SnPPIX (10  $\mu$ M)**



## CHAPTER II

# **Oxidative stress responses and bioenergetic profile of human retinal epithelial cells cultured in normal or high glucose conditions**

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**Abbreviations:** Nrf2, Nuclear factor (erythroid-derived 2)-like 2; DMF, dimethyl fumarate; NG, normal glucose; HG, high glucose; ROS, reactive oxygen species; HO-1, heme oxygenase-1, SnPPIX, tin protoporphyrin IX; GSH, reduced glutathione; BSO, buthionine-sulphoximine, NAC, N-acetylcysteine; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone ;

## Highlights

The Nrf2/HO-1 axis is essential against oxidative stress and inflammatory conditions

Influence of glucose levels on antioxidant response in retinal cells is unknown

High glucose changes glutathione and ROS response but not activation of Nrf2/HO-1

High glucose causes retinal mitochondrial dysfunction

The Nrf2 activator carnosol accelerates wound closure *in vitro*

## Abstract

Retinal pigment epithelial cells exert an important supporting role in the eye and develop adaptive responses to oxidative stress or high glucose levels, as observed during diabetes. Endogenous antioxidant defences are mainly regulated by Nrf2, a transcription factor that is activated by naturally-derived and electrophilic compounds. Here we investigated the effects of the Nrf2 inducers dimethyl fumarate (DMF) and carnosol on antioxidant pathways, oxygen consumption and wound healing in human retinal pigment epithelial cells (ARPE-19) cultured in medium containing normal (NG, 5 mM) or high (HG, 25 mM) glucose levels. Nrf2 nuclear translocation and heme oxygenase activity increased in ARPE cells treated with 10  $\mu$ M DMF or carnosol irrespective of glucose culture conditions. However, high glucose rendered retinal cells more sensitive to regulators of glutathione synthesis or inhibition and caused a decrease of both cellular and mitochondrial reactive oxygen species. Mitochondrial function measured with the Seahorse XF analyzer and ATP production revealed that culture in HG progressively decreased respiration and ATP over time. This effect was accompanied by morphologically damaged mitochondria, as assessed by electron microscopy analysis. Acute treatment with DMF or carnosol did not restore oxygen consumption in HG cells; conversely, the compounds reduced cellular maximal respiratory and reserve capacity, which was completely prevented by N-acetylcysteine thus suggesting a thiol-mediated mechanism. Interestingly, the scratch assay showed that wound closure was faster in cells cultured in HG than NG and was accelerated by carnosol. This effect was reversed by an inhibitor of heme oxygenase activity. In summary, these data indicate that culture of retinal epithelial cells in HG does not affect the activation of the Nrf2/heme oxygenase axis but influences other crucial oxidative and mitochondrial-dependent cellular functions. The additional effect on wound closure suggests that results

obtained in *in vitro* experimental settings need to be carefully evaluated in the context of the glucose concentrations used in cell culture.

**Keywords:** Nrf2 activators, heme oxygenase, glucose levels, retinal cells, mitochondria, oxygen consumption, scratch assay

## 1. Introduction

The retinal pigment epithelium is the cell layer in the retina that plays a crucial role in protection and maintenance of photoreceptors while also mediating the uptake of nutrients, ions and water and eliminating waste from the subretinal tissue [1]. Several age-related diseases of the eye, such as macular degeneration and diabetic retinopathy, are associated with damage of the retinal epithelium which reduces its protective function and likely contributes to exacerbation of the disease process. Hyperglycemia in particular can severely impair all layers of the eye structure as alteration of retinal endothelial cells, pericytes and epithelium function is associated with the development of diabetic retinopathy. Persistent oxidative stress and a chronic inflammatory status of the eye appear to be important factors underlying the complications of diabetic retinopathy [2] and the activities of major antioxidant enzymes, including glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, were decreased in the retina of diabetic rats [3]. In addition, recent experimental evidence strongly implicates mitochondrial dysfunction as a significant mechanistic cause in the damage exerted by high glucose to retinal endothelial cells and pericytes [4].

The retinal epithelium has been shown to possess reliable antioxidant and defensive systems that are controlled by Nrf2, a ubiquitous transcription factor responsible of activating a panel of genes that actively participate in detoxification and survival processes [5,6]. Studies focusing on Nrf2 in the retinal epithelium have demonstrated a central role for this transcription factor in protection against oxidative challenge derived from phototoxic stress and cigarette smoke [6,7]. Heme oxygenase-1 (HO-1) is one key mediator of Nrf2-induced protection, acting via the production of the antioxidants biliverdin/bilirubin and the signalling molecule carbon monoxide from heme degradation [8]. In the eye HO-1 reduces inflammation, promotes corneal wound healing and its overexpression following hemin administration protects the retinas in



streptozotocin-induced diabetic rats [9,10]. From a therapeutic perspective it is important to note that several electrophilic or plant-derived compounds possess the ability of activating the Nrf2/HO-1 axis [11] and reduce inflammation of the retina [12,13], suggesting that the identification of promising molecules that enhance the expression of endogenous antioxidant proteins in retinal tissue may have therapeutic value in the treatment of eye diseases. However, whether the retinal cellular response to Nrf2/HO-1 activators is affected by high glucose is currently unknown.

In this study we show that dimethyl fumarate (DMF) and carnosol, two electrophilic compounds recently shown in microglia cells to be among the best HO-1 inducers with a low cytotoxicity profile [11], similarly activate the Nrf2/heme oxygenase axis in human retinal pigment epithelial cells cultured in normal glucose levels (5 mM) or hyperglycaemic conditions (25 mM). We further demonstrate that hyperglycaemic conditions result in decreased mitochondrial function, reduced reactive oxygen species production and promotion of cell migration in vitro and that these effects can be modulated by Nrf2 activators.

## **2. Materials and Methods**

### *2.1. Chemicals and reagents*

Dimethyl fumarate (DMF) and carnosol were purchased from Sigma. Hemin and tin protoporphyrin IX (SnPPiX) were from Frontier Scientific (Logan, USA). All other chemicals and reagents were from Sigma unless otherwise stated.

### *2.2. Cell culture*

The human retinal pigment epithelial cell line ARPE-19 was purchased from ATCC (USA) and cultured in DMEM:F12 medium containing 10% FBS, 100 U/ml penicillin, 10 µg/ml streptomycin and supplemented with either normal glucose (NG, 5 mM) or high glucose (HG, 25 mM) to reflect physiological or hyperglycemic glucose levels, respectively. Cells were grown in a 5% CO<sub>2</sub> atmosphere at 37°C and were adapted for 1 week to different glucose levels prior to their use in experiments. All experiments and measurements described below were conducted in parallel in NG and HG cultured cells and cells were used between passages 25 to 35.

### *2.3. Preparation of nuclear extract and detection of Nrf2 by Western blotting*

Expression of Nrf2 was determined by Western blotting 2 h after incubation of ARPE-19 cells in medium alone or in the presence of 10  $\mu$ M carnosol or DMF. At the end of the incubation nuclear extracts were isolated using a commercial kit from Actif Motif (BeNGium) following the manufacturer's instructions and protein concentration was measured with the BCA protein assay kit (Thermo Scientific). For Nrf2 detection, 30  $\mu$ g of protein for each sample was separated by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to nitrocellulose membranes; the nonspecific binding of antibodies was blocked with 5% nonfat dried milk and 0.1% Tween in TBS. Membranes were then probed overnight with a rabbit polyclonal antibody against Nrf2 (Santa Cruz Biotechnology, Inc., 1:1000 dilution). Lamin A/C (clone N-18, goat polyclonal, Santacruz Biotechnology) was used as a nuclear control. Membranes were then incubated with secondary antibodies coupled to horseradish peroxidase (goat anti-rabbit, Cell Signaling Technology or donkey anti-goat, Jackson ImmunoResearch) for 1 h at room temperature. Bands were detected with chemiluminescent substrates (RevelBlot<sup>®</sup> Intense, Ozyme) and images captured using a G:Box F3 Imagery Station and GeneSys Software (Syngene, Cambridge, UK).

### *2.4. Heme oxygenase activity assay*

Cells cultured in 100 mm diameter petri dishes were collected 6 or 24 h after incubation with 10  $\mu$ M hemin, carnosol or DMF and assessed for heme oxygenase activity. The assay is based on the spectrophotometric determination of bilirubin as the final product of heme degradation by heme oxygenase [14]. Samples were incubated with the substrate hemin, NADPH, liver cytosol (a source of biliverdin reductase) and other co-factors to sustain heme oxygenase activity. The reaction was allowed to proceed for 1 h at 37°C in the dark and was terminated by addition of chloroform to extract the bilirubin produced. Bilirubin was measured spectrophotometrically as described before and calculated in picomoles bilirubin/mg protein/60 min [15].

### *2.5. Determination of reduced glutathione content*

Reduced glutathione (GSH) was determined in ARPE-19 cells 6 h after exposure to 10  $\mu$ M carnosol or DMF or 1 mM buthionine-sulphoximine (BSO), an inhibitor of glutathione synthesis. GSH was also measured 16 h after incubation with 1 or 2.5 mM N-acetylcysteine (NAC), a precursor of glutathione synthesis. At the end of the treatments, samples collected in 2% w/v sulfosalicylic acid were reacted for 5 min with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB 0.2 mM in 0,3 M sodium phosphate, 10 mM EDTA, pH 7,5), followed by spectrophotometric readings at 412 nm [16]. GSH content was calculated based on a GSH standard curve and results were expressed as  $\mu$ M of GSH over mg of protein.

#### *2.6. Measurement of reactive oxygen species*

Cellular ROS production was measured in cells pretreated for 6 h with 10  $\mu$ M carnosol or DMF. Cells were loaded with 40  $\mu$ M 2',7'-dichlorofluorescein diacetate for 30 min at 37°C and ROS generation was then measured over a period of 2 h and 30 min by a fluorescence plate reader (Tecan, Grödig, Austria) set at 480 nm for excitation and 520 nm for emission. Mitochondrial ROS production was also determined upon addition of antimycin A (50  $\mu$ M), an inhibitor of complex III in the mitochondrial respiratory chain, in the presence or absence of 10  $\mu$ M carnosol or DMF or following 6 h pretreatment with the Nrf2 activators. By blocking complex III, antimycin A prevents the normal flow of electrons through the electron transport chain leading to generation of the superoxide radical.

#### *2.7. Cellular bioenergetic analysis using the Seahorse Bioscience XF analyzer*

Analysis of bioenergetic parameters was performed in intact cells using the XF24 analyzer from Seahorse Bioscience (Billerica, MA, USA). Oxygen consumption rate (OCR), which indicates mitochondrial respiration, and extracellular acidification rate (ECAR), an index of glycolysis, were measured in real time in ARPE cells cultured at the two glucose concentrations. A classical Mito Stress test was performed according to the following procedure: 1) basal respiration was measured in unbuffered medium; 2) oligomycin (1  $\mu$ g/ml final concentration), an inhibitor of ATP synthesis, was injected to determine respiration linked to ATP production; 3) the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.7  $\mu$ M final concentration) was added to measure maximal respiration and 4) antimycin A and rotenone (1  $\mu$ M final) were applied

in combination to block respiration due to simultaneous inhibition of complexes III and I, respectively. An optimal cell density of 50000 cells/well was determined in preliminary experiments. The Mito Stress test was conducted in control cells and cells pretreated for 6 h with 10  $\mu$ M carnosol or DMF, or cells pre-incubated for 14 h with 1 mM NAC prior to exposure for 6 h to carnosol or DMF.

### *2.8. ATP measurements*

ATP production was measured using the ATPLite™ bioluminescent assay kit (Perkin Elmer, Courtaboeuf, France) following the manufacturer's instructions.

### *2.9. Mitochondrial staining*

Determination of mitochondrial mass was performed using acridine orange 10-nonyl bromide, which binds to cardiolipin found in mitochondria. Cells were fixed for 30 min with 4% paraformaldehyde in PBS at 37°C and incubated for 30 min with 1  $\mu$ M acridine orange 10-nonyl bromide at 37°C in the dark. After two washes with PBS, cells were analyzed with flow cytometry using a CyAn™ ADP LX7 Analyzer (Beckman Coulter).

### *2.10 Electron microscopy*

Cells grown in NG or HG were fixed with glutaraldehyde (2.5% in PBS) for 1 h at room temperature. Cells were scraped and centrifuged at 1000 g for 5 min followed by 2 washings with PBS. Samples were processed for electron microscopy by fixing with 1% osmium tetroxide, dehydrated by successive ethanol washes (70%, 90%, 100%, 100%) and impregnated with epoxy resin. After polymerization, sections (80-90 nm) were cut using a Reichert Ultracut S ultramicrotome and stained with 2% uranyl acetate and Reynold's lead citrate. Sections were assessed using a JEOL 1011 transmission electron microscope equipped with a GATAN Erlangshen CCD camera. Cells cultured in NG or HG medium for 1 or 6 weeks were compared in these experiments.

### *2.11. Scratch assay test for determination of cell migration*

ARPE-19 cells grown to confluence in 6-well dishes were scratched with a 1 ml tip and wound closure was followed over time. Closure was monitored under an inverted microscope at 0, 6, 24 and 36 h and photographs were taken for analysis. Quantitative assessment of wound area was performed using the ImageJ software (NIH) and expressed over the area measured at time 0 h. Cells treated with 10  $\mu$ M carnosol or DMF in the presence or absence of SnPPIX (10  $\mu$ M), were used for these experiments.

### *2.12. Data analysis*

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using Graph Pad Prism. One-way ANOVA combined with the Bonferroni or Tukey's multiple comparison tests and the t-test were applied where appropriate. Differences were considered to be significant at  $P<0.05$ .

## **3. Results**

### *3.1. Nrf2 nuclear translocation and heme oxygenase activity are similarly induced in ARPE cells cultured in normal glucose (NG) or hyperglycaemic conditions (HG)*

To investigate whether glucose levels affect the response to Nrf2/HO-1 activators, we examined the effect of carnosol and DMF on Nrf2 protein expression and heme oxygenase activity in ARPE cells cultured in medium containing 5 or 25 mM glucose. We chose these two activators based on a previous screening study conducted by our group in microglia cells [11], where the two compounds emerged as among the best HO-1 inducers with a low cytotoxicity profile. The results of Fig. 1A indicate that both carnosol and DMF induced Nrf2 nuclear translocation and that this effect was similar in NG or HG cultured cells (no significant difference in densitometric analysis, see Fig. 1B). A comparable increase in heme oxygenase activity was also detected in ARPE cells after 6 or 24 h exposure to the two compounds in the different glucose conditions (Fig. 1C and 1D). Hemin was used in our study as a positive control, as it acts as a substrate and inducer of HO-1. Of note, heme oxygenase activity levels remained markedly elevated in cells incubated with carnosol also at 24 h, while the activity declined to basal values in cells treated with DMF.

### *3.2. Culture in hyperglycaemic medium changes the response to modulators of glutathione levels and ROS production*

It is known that electrophilic substances such as DMF and carnosol readily react with cellular nucleophilic centers, including sulphhydryl groups present in GSH, thus leading to transient modulation of GSH levels. We therefore examined GSH content 6 h after incubation with carnosol or DMF as well as the modulators of GSH synthesis BSO and NAC. We first determined that GSH absolute content was markedly lower in HG cells ( $11.82 \pm 0.5$  nmoles,  $n=5$ ) compared to NG ( $15.16 \pm 1.0$  nmoles,  $n=5$ ). However, since protein levels were also lower in HG cells, GSH content expressed over mg of protein was only slightly reduced compared to that of cells cultured in NG (Fig. 2A). We also observed that changes in GSH after incubation with different modulators were much more pronounced in HG than NG cells, with significant increase of GSH after NAC or carnosol and decrease after DMF exposure (Fig. 2A). In general, NG cells exhibited less prominent responses to both GSH inducers and depleting compounds.

Our results obtained with ROS measurements indicated an unexpected phenomenon since both cellular and mitochondrial-derived ROS production were significantly lower in HG compared to NG cultured cells (Fig. 2B and 3). Preincubation for 6 h with carnosol, but not DMF, decreased cellular ROS levels (Fig. 2), while mitochondrial ROS induced by antimycin A was not significantly changed by either carnosol or DMF preincubation or co-incubation during ROS measurement (Fig. 3). The co-incubation approach was adopted to identify potential intrinsic antioxidant properties of carnosol or DMF, which could be evidenced prior to induction of Nrf2-dependent protective enzymes. The inclusion in these experiments of SnPPiX (10  $\mu$ M) to inhibit heme oxygenase activity did not significantly modify the production of ROS.

Thus, prolonged culture of retinal epithelial cells in NG or HG does not affect the Nrf2/HO-1 response triggered by carnosol or DMF while modulation of GSH and ROS production is more readily associated to glucose levels in culture.

### *3.3. Bioenergetic profile of ARPE cells cultured in normal and hyperglycaemic conditions*

The decreased cellular and mitochondrial ROS measured in HG led us to postulate that mitochondrial function could be impaired by supraphysiological glucose levels in the retinal

epithelium. To test this hypothesis we determined cellular bioenergetics by using the Seahorse XF analyzer, a recently developed machine that measures in real time oxygen consumption and glycolysis in intact cells in culture. The sequential use of pharmacological compounds that affect specific activities of mitochondria, such as production of ATP (oligomycin), uncoupling activity (FCCP), and electron flow from complex I and III (rotenone and antimycin A, respectively), allows to generate a bioenergetic profile that directly correlates with cellular energy production based on oxidative phosphorylation and the glycolytic pathway. Fig. 4A shows that cellular respiration (OCR) and the response to modifiers of mitochondria function were markedly lower in cells cultured in HG compared to NG. We also observed a depression of glycolytic activity in HG conditions, as measured by the extracellular acidification rate (ECAR, Fig. 4B). With the results of the MitoStress test we quantified several bioenergetic parameters and we determined that HG caused a significant decrease in basal respiration and in oxygen consumption due to ATP turnover (Fig. 4C). Maximal respiration and respiratory reserve capacity were also markedly reduced by HG, indicating a generally depressed mitochondrial activity in cells cultured in hyperglycaemic conditions.

Measurements of ATP levels in ARPE cells during culture in NG and HG over time revealed that hyperglycemia progressively leads to a decline in ATP production to values that were significantly lower compared to cells cultured in NG (Fig. 5A). Mitochondrial content measured by the use of the dye acridine orange 10-nonyl bromide was consistently higher in cells cultured at HG (Fig. 5B and C). In electron microscopy studies performed to investigate the quality and morphology of mitochondria 1 week and 6 weeks after culture of ARPE cells in NG or HG, we observed the following (Fig. 5D): 1) mitochondria in HG cells exhibited after 1 week of culture changes in morphology, with less evident cristae and mild mitochondrial swelling compared to cells in NG; 2) after 6 weeks of culture, mitochondria in NG cells maintained a healthy morphology while cells in HG were composed of different populations that included cells with apparently normal mitochondria, cells with morphologically damaged mitochondria and cells with no visible mitochondria. No apoptosis or necrosis was detected in cells cultured in HG but increased fat deposits and swelling of the endoplasmic reticulum (supplementary Fig 1) and evidence of altered metabolically activity (lysosomes and glycogen vesicles) were observed.

### *3.4 DMF and carnosol decrease maximal and reserve mitochondrial function: protective role of N-acetylcysteine*

Nrf2 has been recently reported to increase the rate of respiration and the efficiency of oxidative phosphorylation in cells and isolated mitochondria [17]. We therefore tested whether our Nrf2 activators could modify mitochondrial activity and, eventually, reverse the negative effect of HG on respiration. In contrast to this hypothesis, exposing ARPE cells to DMF and carnosol for 6 h did not augment oxygen consumption and we observed a marked decrease in the FCCP-mediated response (Fig. 6A and B). The effect of carnosol was more pronounced compared to DMF and this was reflected in a dramatic decline of maximal respiration and respiratory reserve capacity (Fig. 6E and F). Notably, the thiol donor NAC completely prevented these effects and similar results were obtained in NG (Fig. 6) and HG conditions (Fig. 7), indicating the independency of these processes on glucose culture conditions. No significant changes in ATP levels were observed in these experiments and inhibition of heme oxygenase activity did not affect the bioenergetic profile (data not shown).

### *3.6. Carnosol stimulates wound closure in the scratch assay*

It is known that heme oxygenase promotes wound healing and epithelial cells migration [18-20] and a recent study showed that Nrf2 knockout mice exhibited a delayed healing in the corneal epithelium [21]. Therefore, we examined whether DMF and carnosol could affect wound closure of ARPE cells using the scratch assay. We observed initially that closure of wounds in HG conditions was accelerated compared to NG cultures (Fig. 8). Application of carnosol, but not DMF, at the time of scratch formation resulted in a significantly faster wound closure in NG cultures (Fig. 9A). A similar tendency was detected in HG conditions but this was not statistically significant (Fig. 9B). Interestingly, inhibition of heme oxygenase activity using SnPPiX completely reversed this effect in both NG and HG conditions (Fig. 9C and 9D, respectively). In preliminary experiments we assessed the pro-healing properties of the compounds at 10 and 20  $\mu$ M concentrations; while DMF was ineffective at both concentrations, 20  $\mu$ M carnosol did not further improve wound closure compared to 10  $\mu$ M (data not shown).

## **4. Discussion**



Activators of the Nrf2/HO-1 axis possess interesting pharmacological properties that are relevant for the treatment of inflammatory conditions; one notable example of their application is the recent approval of dimethyl fumarate (DMF) as a drug effective against multiple sclerosis [22] or its use in psoriasis [23]. Considering that Nrf2/HO-1 activators may exert pharmacological actions also in ocular tissue, which is susceptible to damage caused by oxidative stress and diabetes, we examined here the antioxidant response and wound healing capacity of DMF and carnosol, two well-established Nrf2 activators. Importantly, we compared these responses in retinal epithelial cells (ARPE-19) cultured at physiological glucose concentrations (5 mM, NG) or hyperglycaemic conditions (25 mM, HG) to investigate whether glucose levels affect the action of these compounds. We report that DMF and carnosol similarly activated Nrf2 and HO-1 in ARPE cells cultured in LG and HG, while modulation of glutathione and cellular and mitochondrial ROS production were influenced by the different glucose conditions. Moreover, HG caused a profound depression in mitochondrial function compared to LG, indicating a metabolic disruption under HG conditions. Acute treatment with DMF and carnosol significantly decreased maximal mitochondrial respiratory capacity via a mechanism that involves thiol groups; however, the effect was similar in LG and HG. Finally, we show that HG accelerated wound closure of ARPE cells *in vitro* and application of carnosol further enhanced this effect. These data highlight the influence of glucose culture conditions on antioxidant, mitochondrial and metabolic responses of the retinal epithelium.

We examined in the first instance whether Nrf2 and HO-1, which are classically activated by DMF and carnosol in many cell types, would be equally or differentially induced in physiological glucose levels or hyperglycaemic conditions. We observed a comparable activation of both pathways, which suggests that the electrophilic properties of Nrf2 activators are not influenced by glucose concentrations. We also showed that induction of heme oxygenase by carnosol was prolonged over 24 h while induction by DMF was already lost at this later time point, confirming previous data obtained in microglia cells [11]. A distinctive feature of Nrf2 activators is their effect on GSH levels, which can derive from direct chemical reaction with cysteines of GSH, thus reducing total GSH in the cell, and/or by upregulating the expression of GSH synthetic enzymes as part of the electrophilic counterattack response that is dependent on Nrf2 [24]. In our study GSH levels were modulated by both DMF and carnosol but the response was more

pronounced in HG conditions; similarly, a higher increase of GSH was observed in HG when cells were treated with the glutathione precursor NAC. These data suggest that culture of ARPE cells in HG renders them more susceptible to fluctuations in GSH levels, which has implications for the reductive capacity of the cellular milieu. Accordingly, levels of reduced glutathione were diminished while oxidized glutathione was increased in the retina of diabetic rats compared to healthy animals [25]. Interestingly, both cellular and mitochondrial ROS levels were decreased in HG conditions, a finding that was initially surprising as ROS production has been reported to be elevated by HG in retinal cells [26]. However, our protocol was different in that ARPE cells were cultured in NG or HG for at least one week prior to the beginning of any experiments, whereas the study mentioned above measured ROS levels 24 h after exposure to HG. Thus, the timing of our experiments precluded the detection of early adaptations to HG, but rather was a useful model to determine the effect of prolonged culture of retinal cells in hyperglycaemic conditions. This line of reasoning is supported by findings in retinal endothelial cells, where long-term (7 days) exposure to elevated glucose did not change HO-1 expression which was instead increased by an acute 24 h exposure to HG [27].

Since ROS are mainly generated as bioproducts of oxidative phosphorylation, we postulated that lower cellular and mitochondrial ROS in HG may be due to alteration of mitochondrial function following prolonged culture in HG conditions. This was in fact confirmed using the Seahorse XF analyzer, which showed a consistently depressed oxygen consumption, as well as a decreased response to modulators of mitochondrial function, in cells cultured in HG. From the profile of the cellular bioenergetic assay we determined that basal oxygen consumption, respiration linked to ATP turnover, maximal respiratory and spare reserve capacity were all significantly diminished by HG, indicating a potential mitochondrial damaging effect by prolonged exposure to hyperglycemia. Indeed, cellular ATP levels progressively declined over the weeks in ARPE cells cultured in HG, effectively proving the reduced ability of these cells to generate energy. Electron microscopy analysis revealed mildly swollen mitochondria and less defined cristae after 1 week of exposure to HG. Interestingly, after 6 weeks in HG no cell death by apoptosis or necrosis was detected but different populations of cells were evident including cells without visible mitochondria, cells with damaged mitochondria and cells with apparently normal mitochondria. This latter group may represent cells that optimally adapted to HG over

time and the compromised mitochondrial activity in HG likely reflects the combined effect of the different cells populations. Using electron microscopy we could not visibly notice changes in mitochondrial content (this technique is in fact not appropriate for quantification of mitochondria) but mitochondrial mass measured with a dye specific for mitochondria was increased in HG. We interpret this response as a compensatory mechanism developed by HG cells, which attempt to overcome decreased mitochondrial function by augmenting mitochondrial biogenesis. An additional explanation to reduced respiration in HG cells could be that energy production relies more robustly on glycolysis since glucose is abundant in the cell culture medium. However, even the extracellular acidification rate (ECAR), which is an index of glycolysis, was lower in cells cultured in HG compared to LG. Since pyruvate (the last product of glycolysis) is used as a substrate of respiration, decreased glycolysis may be a direct consequence of less substrate requirement by HG-damaged/malfunctional mitochondria. Alternatively, prolonged HG conditions also result in inhibited glycolysis. It is important to note that recent studies have shown dramatic changes in mitochondrial morphology and decreased oxygen consumption also in retinal endothelial cells [28,29] and pericytes [4] exposed to HG, indicating the high susceptibility of retinal tissue to HG-mediated mitochondrial damage that has been linked to the development of diabetic retinopathy [30].

We also show that maximal respiratory and reserve capacity were especially attenuated by carnosol and DMF after 6 h incubation. This effect was independent of glucose culture conditions and completely prevented by pre-incubation with the glutathione precursor NAC. A decrease in maximal and reserve capacity may be indicative of mitochondrial damage and the protection by NAC strongly suggests that an oxidative/thiol-mediated mechanism is involved. Since mitochondria contain cysteine residues that are highly susceptible to modification by oxidative stress, it is tempting to speculate that loss of maximal respiration is due to electrophilic attack of specific cysteines in the respiratory complexes by Nrf2 activators. Conversely, the presence of NAC provides a powerful shield for protection of these highly sensitive cysteines. We also noted that NAC prevents HO-1 induction by electrophiles [11,16], suggesting some potential links between HO-1 upregulation and modification of mitochondrial function by these compounds. To our knowledge, inhibition of mitochondrial maximal and reserve respiratory capacity by Nrf2 activators has never been described before and we

hypothesize that modification of mitochondrial function may explain some of the undesired side effects of these compounds.

To further study pharmacological actions of Nrf2 activators relevant to retinal tissues, we assessed wound closure *in vitro* using the scratch assay. Interestingly, HG accelerated wound closure, which was unexpected since it is known that wound healing is compromised in the diabetic retina [2]. However, other reports have described this phenomenon in retinal endothelial cells in culture [31] and clearly the *in vitro* situation does not simulate the *in vivo* setting where the participation of other cell types, including inflammatory cells, contributes to wound healing. In our experiments application of carnosol further stimulated wound closure and inhibiting heme oxygenase activity abolished this effect. Since carnosol promoted a long-lasting increase in heme oxygenase activity, while the effect of DMF was of shorter duration, we suggest that this may be the key difference between the pro-healing property of carnosol compared to DMF.

In conclusion, our findings demonstrate that glucose concentrations in culture significantly modulate oxidative and metabolic cellular responses to Nrf2 activators in the retinal epithelium. Of particular note is the depression of mitochondrial function and energy production in ARPE cells by HG conditions, which could not be rescued by acute exposure to inducers of Nrf2; however, it would be interesting to examine whether long-term treatment with these compounds prevents this effect. As the Nrf/HO-1 axis is being considered for therapeutic drug discovery [8], the data presented here highlight the importance of monitoring pharmacological actions of Nrf2 activators in conditions that simulate the pathologic states associated with impaired glucose metabolism and hyperglycemia.

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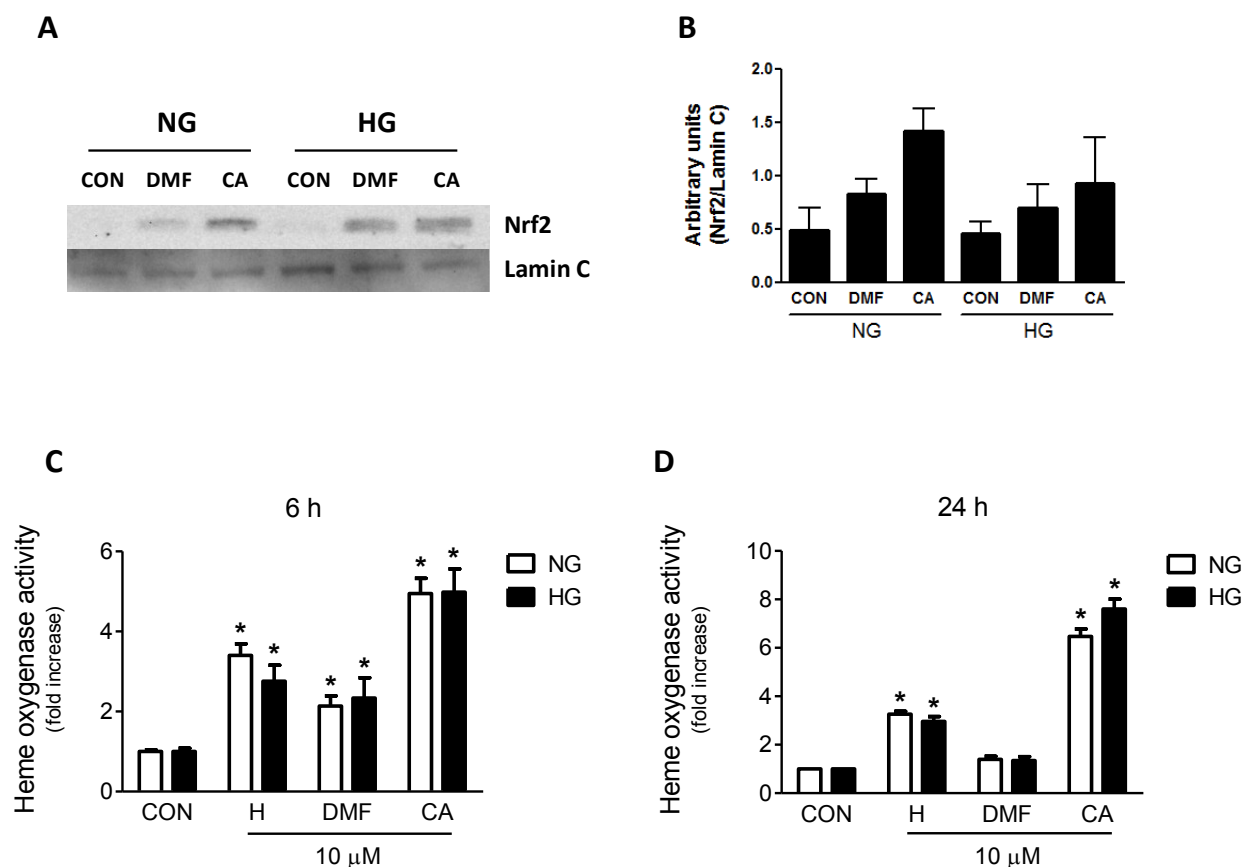
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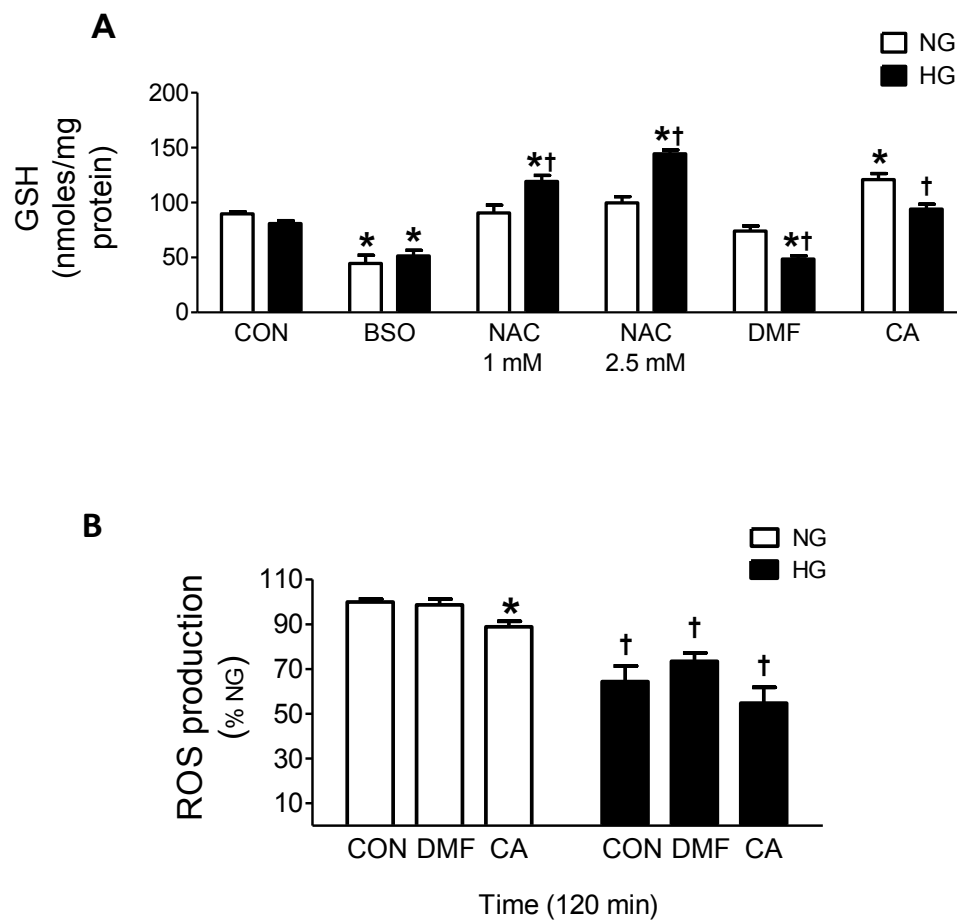
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**FIGURE 1.** Effect of DMF and carnosol on Nrf2 nuclear translocation and cellular heme oxygenase activity in ARPE-19 cells grown in normal (NG) or high (HG) glucose. A) Nrf2 expression measured by Western blotting in nuclear extracts of cells 2 h after treatment with 10  $\mu$ M DMF or carnosol. Blot is representative of 3 independent experiments and in B) are the results of the densitometric analysis. C) and D) Heme oxygenase activity measured 6 and 24 h after incubation with 10  $\mu$ M hemin, DMF or carnosol. No differences in these responses were observed in LG or HG. Data are the mean  $\pm$  SEM of 4-6 independent experiments per group. \*  $P < 0.05$  vs. control (CON).

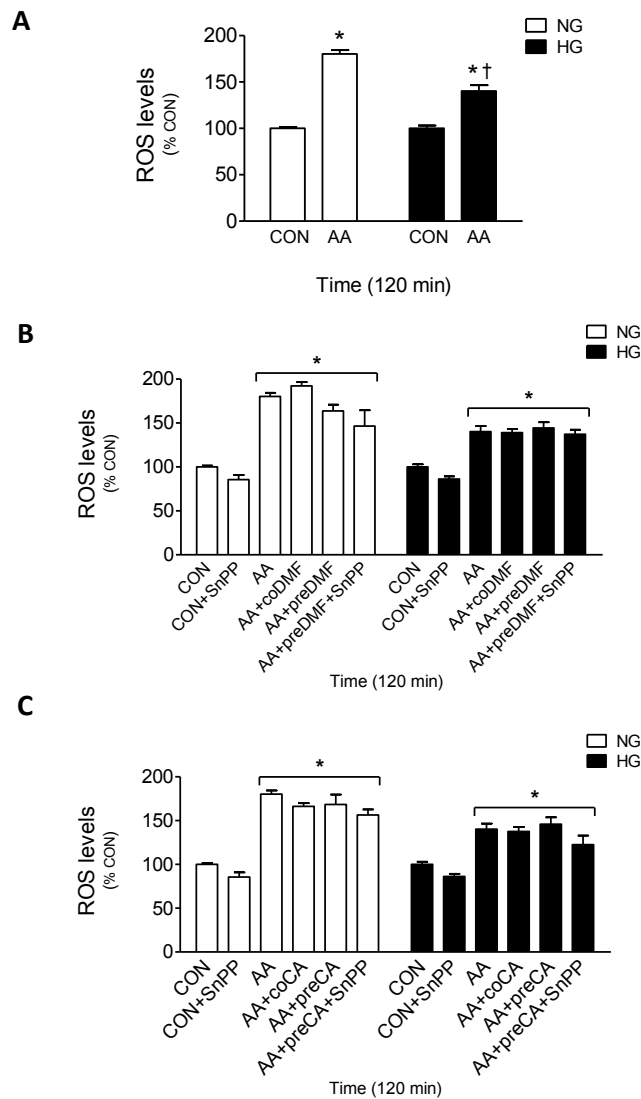




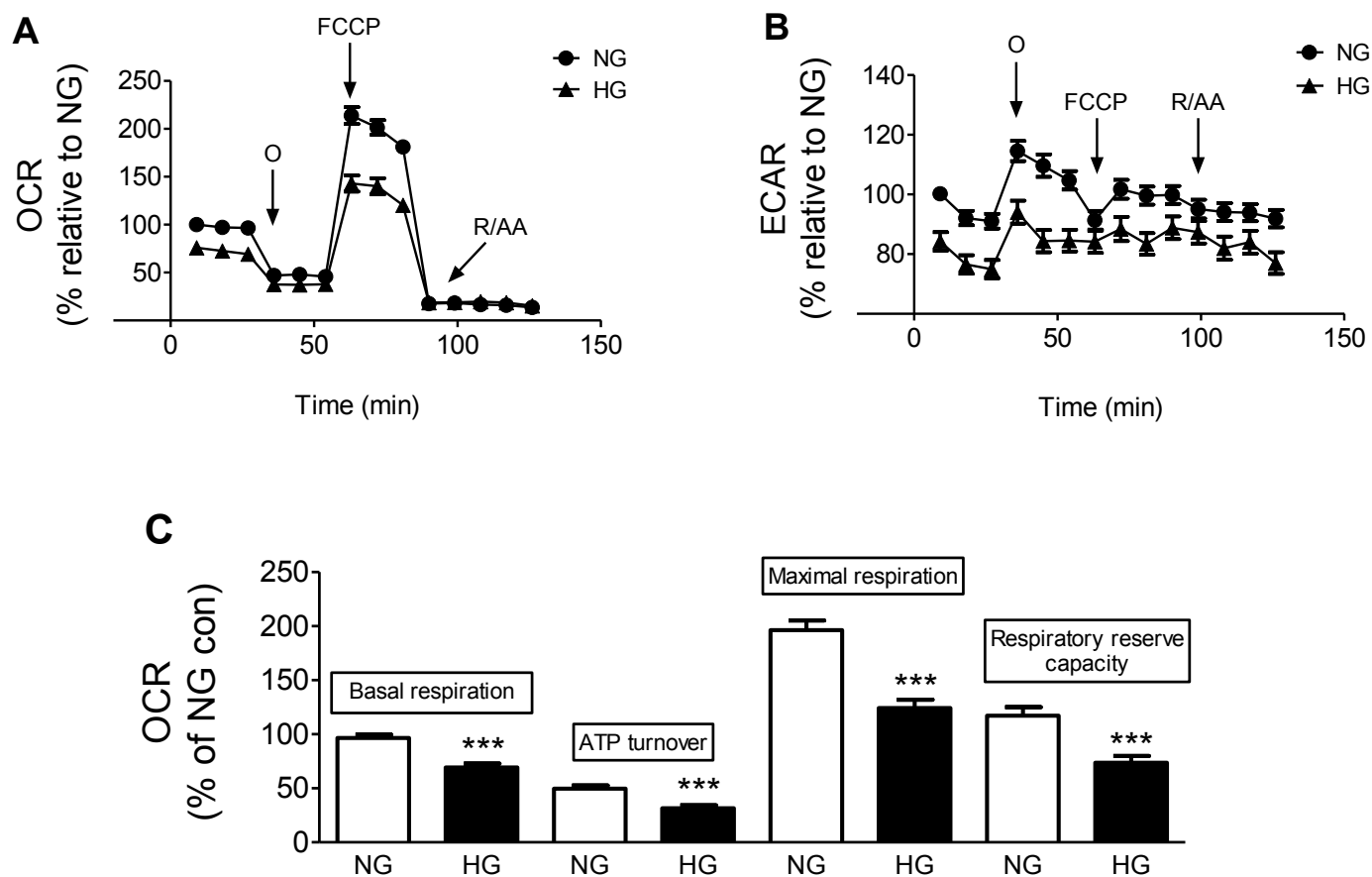
**FIGURE 2.** Changes in reduced glutathione (GSH) and ROS levels in ARPE-19 cells grown in normal (NG) or high (HG) glucose and exposed to DMF or carnosol. A) Cells were treated with the inhibitor of glutathione synthesis BSO (1 mM), the glutathione precursor NAC (1 and 2.5 mM) or 10  $\mu$ M DMF or carnosol and GSH was assessed by a spectrophotometric assay. B) ROS measurement using a fluorescent dye 6 h after exposure to DMF or carnosol. The basal production of ROS is lower in HG conditions. Data are the mean  $\pm$  SEM of at least 3 independent experiments per group. \*  $P < 0.05$  vs. control (CON);  $^\dagger P < 0.05$  vs. NG.



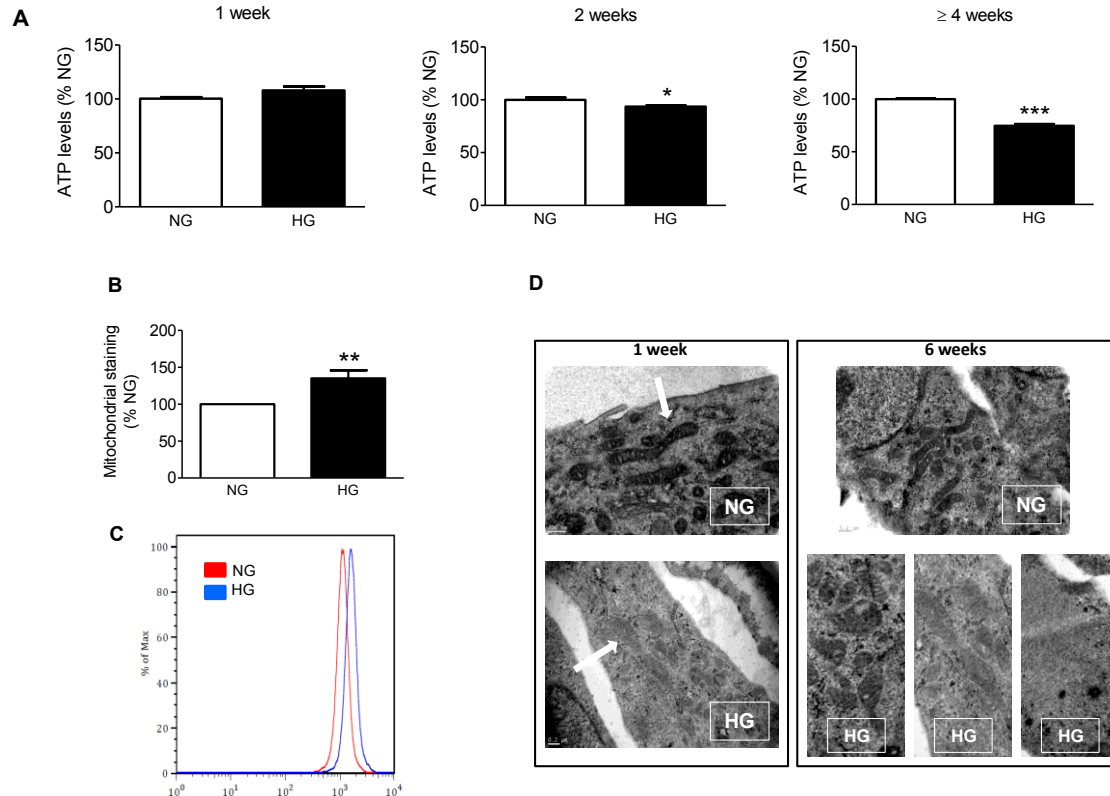
**FIGURE 3.** Mitochondrial ROS production is lower in ARPE-19 cells cultured in high glucose (HG). Mitochondrial ROS generation was stimulated in cells by antimycin A (AA, 50  $\mu$ M), an inhibitor of complex III in the respiratory chain. A) ROS levels are increased by antimycin A in LG and HG conditions but the extent is lower in HG. B) and C) Cells were pre-treated (6 h) or co-treated with 10  $\mu$ M DMF or carnosol and antimycin A for measurement of mitochondrial ROS. In some experiments the inhibitor of heme oxygenase activity SnPPiX (10  $\mu$ M) was utilized. Pre- or co-incubation with the Nrf2 activators did not diminish ROS levels and the heme oxygenase inhibitor did not change these effects. Data are the mean  $\pm$  SEM of at least 4 independent experiments per group. \*  $P < 0.05$  vs. control (CON),  $^{\dagger} P < 0.05$  vs. NG.



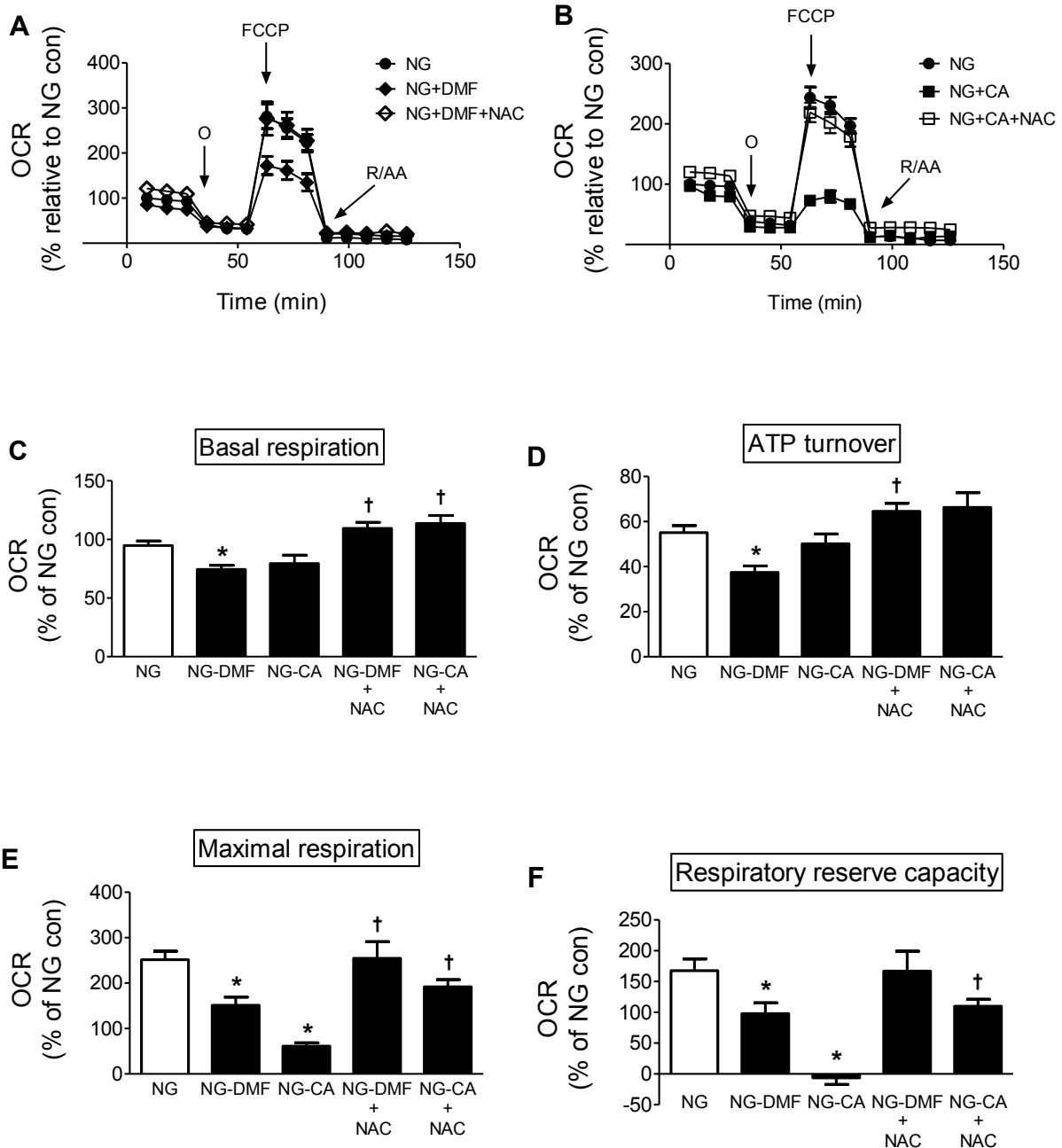
**FIGURE 4.** Mitochondrial function is depressed in ARPE-19 cells cultured in high glucose (HG). The bioenergetic profile of cells grown in NG and HG was determined with the XF analyzer. A) and B) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), an index of glycolysis, in cells treated with oligomycin (O, 1  $\mu\text{g/ml}$ ), FCCP (0.7  $\mu\text{M}$ ) and rotenone and antimycin A (R and AA, respectively, 1  $\mu\text{M}$ ). C) Basal respiration, ATP turnover, maximal respiratory and reserve capacity are all significantly reduced in HG conditions compared to NG. Data represent the mean  $\pm$  SEM of >5 independent experiments per group. \*\*\*  $P < 0.05$  vs. NG.



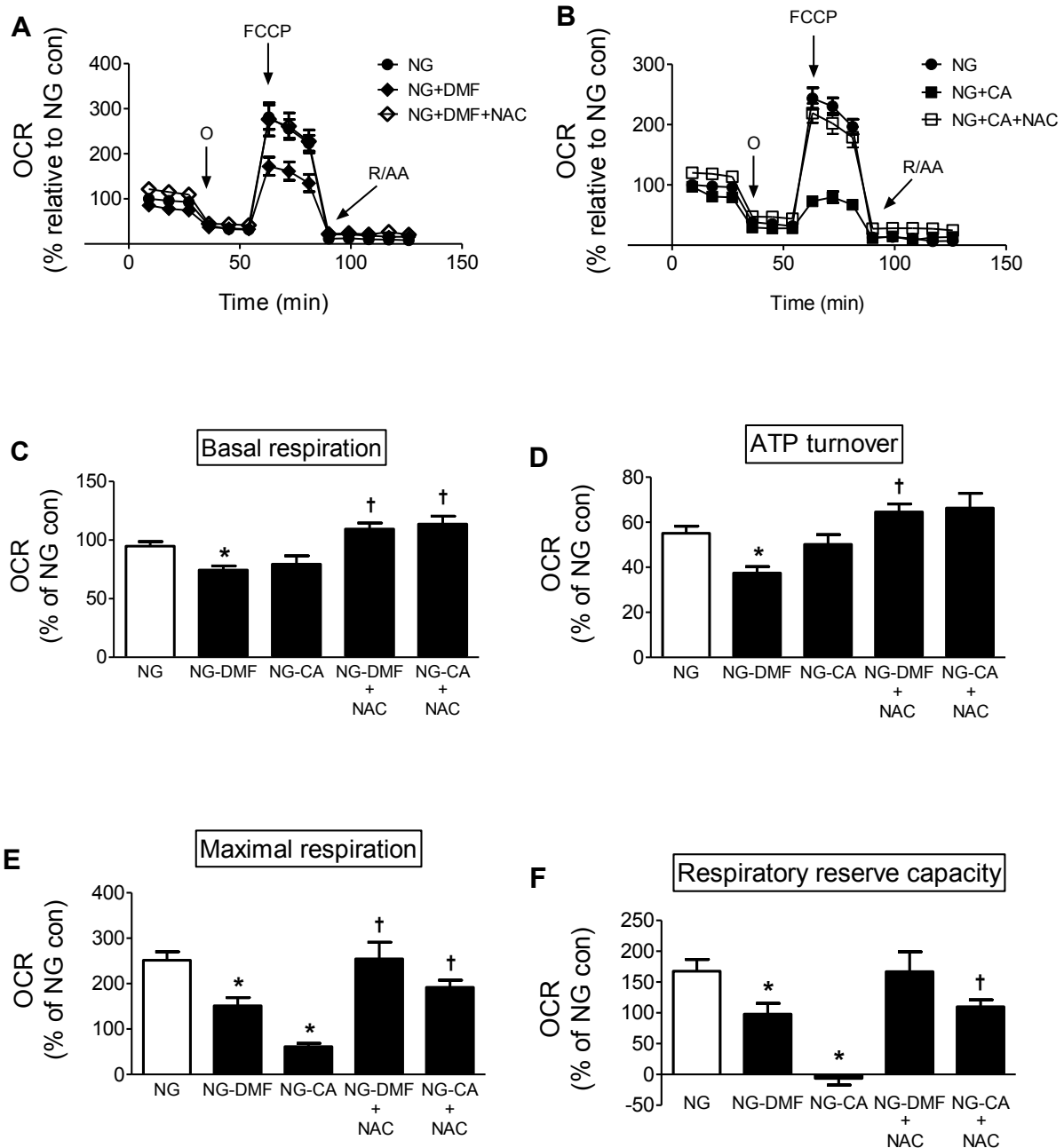
**FIGURE 5.** ATP production, mitochondrial mass and morphology in ARPE-19 cells cultured in normal (NG) and high (HG) glucose. A) ATP production progressively declined over the weeks in cells cultured in HG. B) Mitochondrial mass measured with a mitochondrial-specific dye was higher in cells with HG. C) Transmission electron microscopy images of cells cultured for 1 or 6 weeks in NG or HG. Arrows indicate healthy (NG) or mildly swollen mitochondria with poorly defined cristae (HG). At 6 weeks mitochondria in NG appeared healthy while in HG the co-presence of cells with healthy mitochondria (bottom left panel), damaged mitochondria (bottom center panel) and cells without apparent mitochondria (bottom right panel) was observed. Scale bar = 0.2  $\mu$ m. Data represent the mean  $\pm$  SEM of at least 3 independent experiments per group. \*, \*\*, \*\*\*  $P < 0.05$  vs. NG.



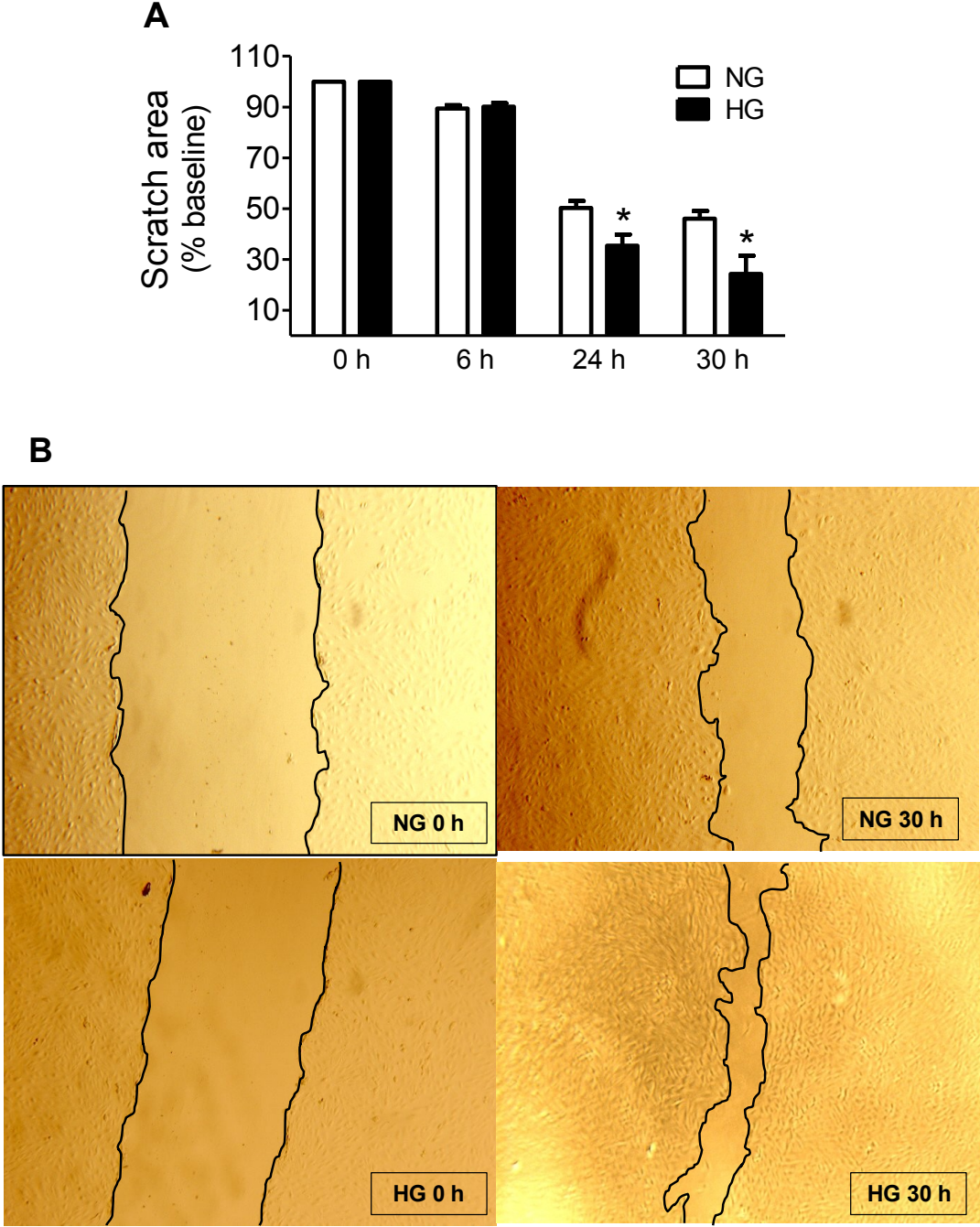
**FIGURE 6.** DMF and carnosol reduce maximal respiration and reserve capacity in cells cultured in normal glucose (NG). A) and B) Oxygen consumption rate (OCR) was assessed in cells 6 h after incubation with 10  $\mu$ M DMF or carnosol. Maximal and reserve respiratory capacities were significantly decreased by both compounds but pre-incubation with NAC (1 mM) completely reversed this effect. Data represent the mean  $\pm$  SEM of at least 3 independent experiments per group. \*  $P < 0.05$  vs. NG CON.



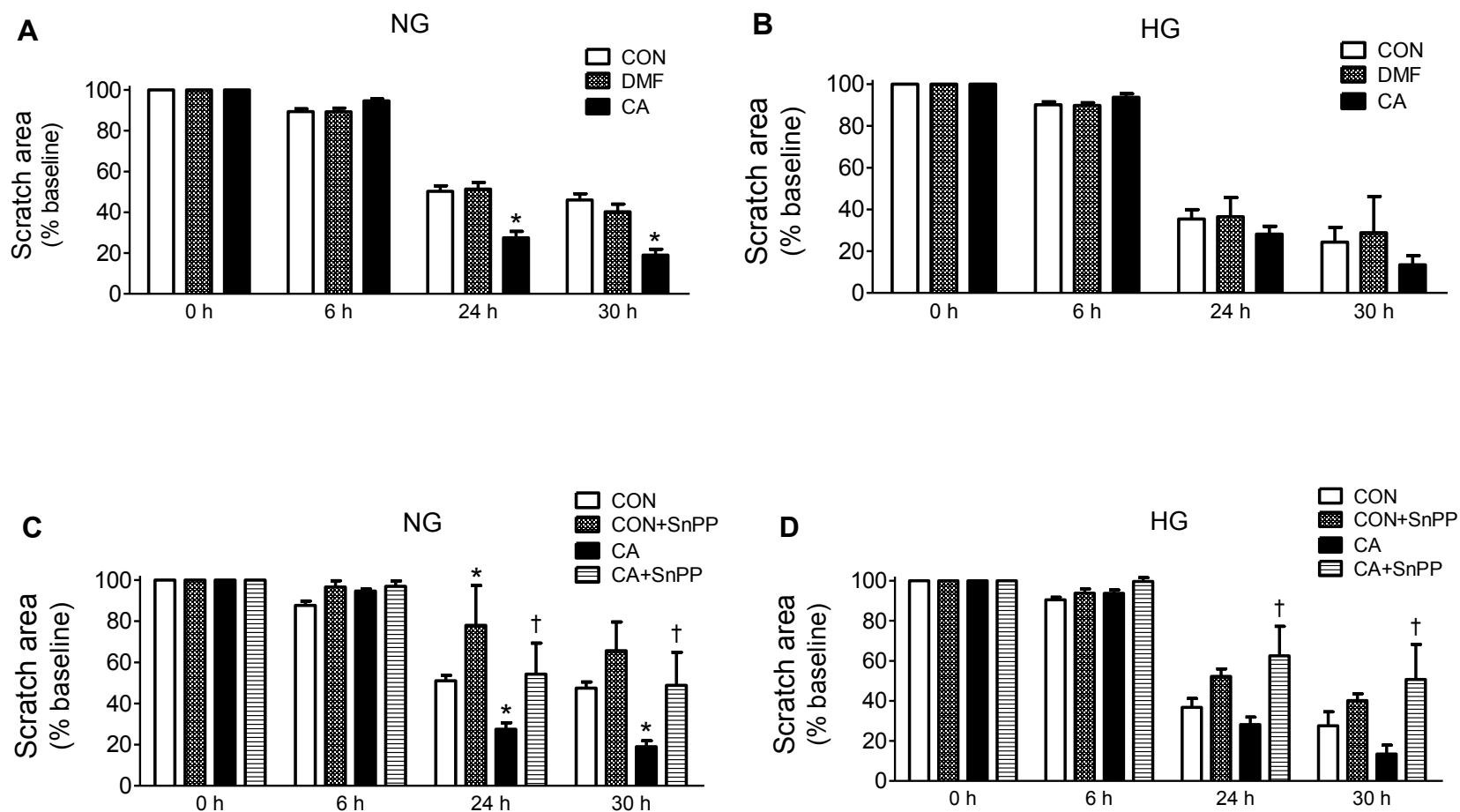
**FIGURE 7.** DMF and carnosol reduce maximal respiration and reserve capacity in cells cultured in high glucose (HG). A) and B) Oxygen consumption rate (OCR) was assessed in cells 6 h after incubation with 10  $\mu$ M DMF or carnosol. Maximal and reserve respiratory capacities were significantly decreased by both compounds but pre-incubation with NAC (1 mM) completely reversed this effect. The inhibitory activity of DMF and carnosol on the FCCP-mediated response was similar between cells in NG and HG. Data represent the mean  $\pm$  SEM of at least 3 independent experiments per group. \*  $P < 0.05$  vs. NG CON.



**FIGURE 8.** High glucose (HG) conditions accelerate wound closure after scratch formation. A scratch obtained with a pipette tip was followed over time and wound closure calculated in reference to the wound area measured at time 0 h. HG conditions accelerated wound closure compared to normal glucose (NG). Data represent the mean  $\pm$  SEM of at least 4 independent experiments per group. \*  $P < 0.05$  vs. NG.



**FIGURE 9.** Carnosol stimulates wound closure in normal (NG) and high (HG) glucose conditions. Wound closure was followed over time in cells cultured in NG or HG conditions in the presence or absence of 10  $\mu$ M DMF or carnosol. Carnosol, but not DMF, markedly accelerated wound closure in LG and HG. Inhibition of heme oxygenase activity by SnPPIX completely reversed this effect. Data are the mean  $\pm$  SEM of at least 4 independent experiments per group. \*  $P < 0.05$  vs. control (CON); †  $P < 0.05$  vs. carnosol.





**Supplementary Figure 1.** Retinal cells cultured in HG exhibit changes linked to altered metabolism. Transmission electron microscopy images of swollen endoplasmic reticulum (top panel) and lipid deposits in vesicles (bottom panel) caused by HG culture. Scale bar= 0.2  $\mu\text{m}$ .



## GENERAL DISCUSSION AND CONCLUSIONS

The search of anti-inflammatory drugs is always ongoing as inflammation, accompanied by oxidative stress, is now recognized as a major underlying component of many chronic diseases affecting the aging population. To progress in this endeavor it is fundamental to identify novel pharmacologically-sensitive pathways that are important in counteracting the disease state and can be manipulated using exogenous substances. The Nrf2/HO-1 axis has been studied in the context of oxidative stress, inflammation and diabetes, and the body of the existing literature strongly supports the significant role of these interconnected systems in tissue protection and restoration of cellular homeostasis.

A most attractive feature of these pathways is that the knowledge of their regulation and function in tissue is sufficient, albeit not complete, for the identification and design of chemical entities that can be used as pharmacological agents in models of disease. Thus, the electrophilic nature of a growing number of naturally-derived compounds (with sulforaphane and curcumin among the most popular and investigated molecules) is found to be a pre-requisite for the activation of Nrf2 and consequent induction of phase II detoxification genes that include the antioxidant HO-1 and many glutathione-modulating enzymes. The fact that the electrophilicity observed among plant compounds is critical in the activation of mammalian defensive systems indicates the conservative approach of nature in the preservation of useful chemical scaffolds [7,163]. This concept is corroborated by the finding that fumarate, a metabolite of the citric acid cycle and therefore an endogenously produced substance, possesses an electrophilic feature and has been shown to induce Nrf2, HO-1 and to protect the heart against ischemia-reperfusion damage [18]. Remarkably, dimethyl fumarate, a synthetic derivative of fumarate, has been used for many years as an oral agent for the treatment of psoriasis (a classical inflammatory condition of the skin) and has been approved in 2013 (commercial name: *Tecdifera*) for use in multiple sclerosis, a severe inflammatory disease of the nervous system for which no drug was available so far. In view of their high reactivity with cysteine residues, electrophilic moieties can exert favorable effects by activating Nrf2 but also negative outcomes due to their binding with and depletion of glutathione, the most important constitutive antioxidant found in the cell. In addition, also cysteine groups present in many proteins and often regulating their function can be targeted by electrophiles. Thus, the aspecific attack of electrophiles towards cysteine-

rich intracellular targets has to be counterbalanced by their beneficial action obtained via the activation of Nrf2. In practice this means that Nrf2 activators can be used to elicit protective effects only within a small range of concentrations, usually in the low micromolar range. Considering the variety of compounds possessing electrophilic scaffolds, it is also important to select them according to their potency in the induction of protective systems versus their cytotoxic effects. The work presented in this thesis is a step forward in this direction by demonstrating that carnosol, curcumin and dimethyl fumarate are the best small molecules activators of HO-1 with a low cytotoxicity profile in BV2 murine microglia (Chapter I). This result was obtained after screening 56 compounds selected from the literature and systematically testing their effect on HO-1 expression at 6 and 24 hours and evaluating cell damage with increasing concentrations of all the compounds. The capacity to stimulate bilirubin production by certain compounds, thus showing that increased HO-1 is reflected in augmented production of its protective products, is another information that can determine the choice of compound to use in pharmacological studies. I also confirmed the anti-inflammatory properties of the compounds and the involvement of the Nrf2/HO-1 axis in this effect, in line with results published in the literature [15,82,89,164]. Although the present work was focused on the measurement of pro-inflammatory mediators, it would be interesting to assess in future investigations whether electrophiles are able to shift the profile of inflammatory cells from M1 to M2 phenotype, indicating a potential role of the molecules in promoting resolution of inflammation.

Since the possible use of these molecules includes conditions linked to diabetes, it was important to verify their pharmacological action in normal and high glucose conditions (Chapter II). For this purpose I chose retinal pigment epithelial cells, a cell type known to be damaged by hyperglycemia and during diabetes. The studies using carnosol and dimethyl fumarate show that high glucose changes the cellular glutathione and ROS response but not the activation of Nrf2/HO-1 by the compounds. Furthermore, this work demonstrated that high glucose causes retinal mitochondrial dysfunction, in accordance with recent published findings in retinal endothelial cells and pericytes [160,161] and highlighting the susceptibility of retinal cells to glucose-mediated cell damage affecting the mitochondria. Short-term incubation with dimethyl fumarate and carnosol did not reverse mitochondrial dysfunction but instead caused a decrease in maximal respiration in a mechanism that likely engages thiol groups, as the thiol donor N-acetylcysteine prevented this effect. The previously

unknown inhibition of respiration by electrophiles and the involvement of thiols suggest that cysteine groups in the mitochondria are susceptible to attack by these compounds, confirming their aspecificity and their potential deleterious actions on intracellular compartments as commented above. The fact that carnosol accelerated wound closure is indicative of novel applications of this electrophile in the context of wound healing and diabetes complications such as retinopathy.

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## LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

### Publications

- Med Gas Res. 2012 Nov 21;2(1):28. doi: 10.1186/2045-9912-2-28. *Emerging concepts on the anti-inflammatory actions of carbon monoxide-releasing molecules (CO-RMs)*. Motterlini R, Haas B, Foresti R.
- Pharmacol Res. 2013 Feb;68(1):108-17. doi: 10.1016/j.phrs.2012.12.001. Epub 2012 Dec 17. *Vasorelaxing effects and inhibition of nitric oxide in macrophages by new iron-containing carbon monoxide-releasing molecules (CO-RMs)*. Motterlini R, Sawle P, Hammad J, Mann BE, Johnson TR, Green CJ, Foresti R.
- Future Med Chem. 2013 Mar;5(4):367-9. doi: 10.4155/fmc.13.10. *CO-releasing molecules: avoiding toxicity and exploiting the beneficial effects of CO for the treatment of cardiovascular disorders*. Foresti R, Motterlini R.
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- Pharmacol Res. 2014 Mar;81:1-9. doi: 10.1016/j.phrs.2014.01.001. Epub 2014 Jan 13. *Isothiocyanate-cysteine conjugates protect renal tissue against-cisplatin induced apoptosis via induction of heme oxygenase-1*. Tayem Y, Green CJ, Motterlini R, Foresti R.
- Vascul Pharmacol. 2014 Sep;62(3):132-3. doi: 10.1016/j.vph.2014.05.006. Epub 2014 May 23. *Heme oxygenase-1 in diabetic vascular dysfunction*. Foresti R, Motterlini R.
- Chemistry. 2014 Nov 3;20(45):14698-704. doi: 10.1002/chem.201403901. Epub 2014 Sep 15. *Design and synthesis of new hybrid molecules that activate the transcription factor Nrf2 and simultaneously release carbon monoxide*. Wilson JL, Fayad Kobeissi S, Oudir S, Haas B, Michel B, Dubois Randé JL, Ollivier A, Martens T, Rivard M, Motterlini R, Foresti R.
- J Cell Physiol. 2014 Oct 9. doi: 10.1002/jcp.24844. [Epub ahead of print]. *Permanent culture of macrophages at physiological oxygen attenuates the antioxidant and immunomodulatory properties of dimethyl fumarate*. Haas B, Chrusciel S, Fayad-Kobeissi S, Dubois-Randé JL, Azuaje F, Boczkowski J, Motterlini R, Foresti R.
- Biochem Pharmacol. 2014 Oct 29;92(4):661-668. doi: 10.1016/j.bcp.2014.10.011. [Epub ahead of print]. *Regulation of vascular tone in rabbit ophthalmic artery: Cross talk of endogenous and exogenous gas mediators*. Salomone S, Foresti R, Villari A, Giurdanella G, Drago F, Bucolo C.

### Conference proceedings

- Poster: 'SO28, a novel compound that induces cellular heme oxygenase-1 and simultaneously releases carbon monoxide'. JL Wilson, S. Fayad-Kobeissi, O. Souhila, B.

Michel, JL Dubois-Randé, T. Martens, M. Rivard, R. Motterlini and R. Foresti. 5<sup>th</sup> *International Symposium on Nutrition, Oxygen Biology & Medicine*, Paris, June 5-7, 2013.

- Poster: '*Dimethyl fumarate differentially modulates antioxidant and anti-inflammatory responses in murine macrophages cultured under atmospheric or physiological oxygen tension*'. B. Haas, S. Fayad-Kobeissi, S. Chrusciel, J. Boczkowski, JL Dubois-Randé, R. Foresti and R. Motterlini. 5<sup>th</sup> *International Symposium on Nutrition, Oxygen Biology & Medicine*, Paris, June 5-7, 2013.
- Poster: '*Pharmacological activities of CORM-401, a redox sensitive carbon monoxide-releasing molecule, in H9C2 cardiomyocytes*'. S. Fayad-Kobeissi, JL Wilson, B. Michel, JL Dubois-Randé, R. Motterlini and R. Foresti. *Printemps de la Cardiologie*, Strasbourg, April 24-25, 2014.
- Poster: '*HYCOs: Novel Hybrid Molecules that Activate the Transcription Factor Nrf2 and Simultaneously Release Carbon Monoxide*'. JL Wilson, S. Fayad-Kobeissi, O. Souhila, B. Haas, B. Michel, JL Dubois-Randé, A. Ollivier, T. Martens, M. Rivard, R. Roberto and R. Foresti. 8<sup>th</sup> *International Conference on Heme oxygenases, Bioiron & Oxidative Stress*, Sydney, Australia, October 8-11, 2014.
- Poster: '*Carbon Monoxide-Induced Modulation of Mitochondrial Bioenergetics in Bv2 Microglia Cells*'. JL Wilson, BE Mann, JL Dubois-Randé, R. Roberto and R. Foresti. 8<sup>th</sup> *International Conference on Heme oxygenase Symposium on Nutrition, Oxygen Biology & Medicine*, Sydney, Australia, October 8-11, 2014.