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Heme modulates smooth muscle cell proliferation and migration via NADPH oxidase: A counter-regulatory role for heme oxygenase system

J.A. Moraes [a], P. Barcellos-de-Souza [a], G. Rodrigues [a], V. Nascimento-Silva [a], S.V. Silva [a], J. Assreuy [b], M.A. Arruda [a,c], C. Barja-Fidalgo [a,*]

[a] Department of Cell Biology, Universidade do Estado do Rio de Janeiro, UERJ, Rio de Janeiro, RJ, Brazil
[b] Department of Pharmacology, Universidade Federal de Santa Catarina, UFSC, Florianópolis, SC, Brazil
[c] Pesquisa e Inovação, Farmanguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil

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ABSTRACT

Accumulation of vascular smooth muscle cells (VSMC) in response to inflammatory stimuli is a key event in atherogenesis, which commonly occurs in sinuous vessels with turbulent blood flow what leads to hemolysis and consequent free heme accumulation, a known pro-oxidant and pro-inflammatory molecule.

In this work, we investigated the effects of free heme on VSMC, and the molecular mechanisms underlying this process.

Free heme induces a concentration-dependent migration and proliferation of VSMC which depends on the production of reactive oxygen species (ROS) derived from NADPH oxidase (NADPHox) activity. Additionally, heme activates redox-sensitive proliferation-related signaling routes, such as Mitogen Activated Protein Kinase (MAPK) and NF-κB, and induces Heme Oxygenase-1 (HO-1) expression. NADPHox-dependent proliferative effect of heme seems to be endogenously modulated by HO since the pretreatment of VSMC with HO inhibitors potentiates heme-induced proliferation and, in parallel, increases ROS production. These effects were no longer observed in the presence of heme metabolites, carbon monoxide and biliverdin.

The data indicate that VSMC proliferation induced by heme is endogenously modulated by a critical counter-regulatory crosstalk between NADPHox and HO systems.

1. Introduction

Cardiovascular diseases represent the major cause of mortality and morbidity in western countries. Among these conditions, atherosclerosis is the most prominent one. The first step in atherogenesis is the vascular inflammation, which is characterized by oxidized LDL infiltration and foam cell formation. This process is accompanied by endothelial cell lesion, extracellular matrix exposure, platelets aggregation, adhesion of leukocytes and, mainly, by vascular smooth muscle cell (VSMC) accumulation [1]. The VSMC dysfunction is characterized by an exacerbation of cell migration and proliferation, events which are amplified through the release of inflammatory mediators [2–4]. VSMC dysfunction has been implicated in the pathogenesis not only of atherosclerosis, but also of other cardiovascular pathological situations, such as restenosis. The exacerbated activation of VSMC is a key event in the fibrous cap formation (atherosclerosis) or in the neointima formation (restenosis). Notably, these cardiovascular diseases occur mainly in sinuous vessels, and are associated with turbulent blood flow, which may lead to hemolysis and consequent release of free heme [5]. Importantly, our group, and others, has characterized heme as a strong pro-inflammatory molecule to neutrophils, macrophages an endothelium, in which most effects are mediated by ROS derived from NADPHox complex activation [6–10].

The oxidative stress that leads to an increased ROS production via NADPHox activation has been implicated in many cardiovascular disorders [11]. The production of ROS is positively regulated by many of the cytokines whose expression is increased after vascular injury, and also by oscillatory shear stress and mechanical disruption [12]. On the other hand, low levels of ROS have been shown to be necessary for the activation of redox-sensitive signaling pathways that influence many physiological processes [13].

* Corresponding author. Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Av. 28 de Setembro 87 fds, Rio de Janeiro, RJ 20550-030, Brazil. Tel./fax: +55 21 2587 6398.
E-mail address: barja-fidalgo@uerj.br (C. Barja-Fidalgo).

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Besides its ability in activating the NADPHox system, heme is also able to induce the HO system, which is responsible for its metabolization into carbon monoxide (CO), iron and biliverdin, which can be converted to bilirubin by biliverdin reductase. This system is comprised of the constitutive isoform, HO-2, and by the inducible isoform, HO-1 [14]. HO-2 constitutively metabolizes endogenous heme and evidences have shown that its deletion increases ROS accumulation [15]. HO-1 is a stress-responsive enzyme, whose induction by free heme and other stimuli, such as pro-inflammatory molecules, increases the rate of heme catabolism. The by-products of HO-1–mediated heme catabolism have established antioxidant and anti-inflammatory properties [16].

In this scenario, free heme emerges as a putative molecule able to activate key molecular mechanisms in vascular cells that contribute to several cardiovascular disorders. In this work we aimed to elucidate how the pro-and antioxidant properties of heme could modulate VSMC migration and proliferation and the mechanisms underlying its effects.

2. Methods

2.1. Cell culture

A7r5 vascular smooth muscle cells obtained of rat thoracic aorta were originally from the American Type Culture Collection (Rockville, MD USA). Cell culture is better explained in Supplementary methods.

2.2. Cell migration

Chemotaxis assay was accomplished in a modified Boyden Chamber after 4 h of migration. This assay is better described in Supplementary methods.

2.3. Cell proliferation

Cell proliferation was measured by Thymidine [H^3] incorporation after 48 h of treatment. This assay is better described in Supplementary methods.

2.4. ROS production

ROS production was measured using the DHR and DCF probes. Intracellular DHR oxidation was examined under an Olympus IX71 inverted microscope (Tokyo, Japan) equipped for epifluorescence and DCF oxidation was monitored in the Victor^3 Mutilabel fluorescent plate reader (PerkinElmer, Waltham, MA USA). ROS assays are better described in Supplementary Methods.

2.5. Western blot analysis

Total protein content in the cell extracts was determined by the BCA method, except on immunopreciptates samples. Samples were submitted to electrophoresis and transfered to PVDF membranes. Immunoreactive proteins were visualized by the ECL detection. Western blotting is better described in Supplementary methods.

2.6. Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Bonferroni's *t*-test, and $P < 0.05$ was taken as statistically significant. ROS assay (CM-H_2-DCFDA) was analyzed by two-way ANOVA, followed by t-student test in each point.

3. Results

3.1. Heme induces VSMC migration and proliferation via NADPHox

Firstly we investigated heme effects on the two main VSMC functions usually altered in cardiovascular diseases: migration and proliferation. As can be observed in Fig. 1, heme has a potent chemotactic activity on VSMC (Fig. 1A), and is also a potent inducer of VSMC proliferation (Fig. 1B). For both cell functions the effect of heme peaked at 10 μM, a concentration that is believed to be reached in regions of turbulent blood flow, and can be found in the plasma of patients during hemolytic episodes such as such as severe hemolytic crisis in sickle cell disease (up to 20 μM heme) or thalassemia (50–280 μM heme) [10,17]. Heme has shown effects similar to Ang II, used as positive control [18] for migration, but was more potent than this prototypical vasoactive agent in inducing VSMC proliferation. Heme effects were shown to be redox-sensitive, since the antioxidant Trolox™ (100 μM) abrogated heme-induced VSMC migration and proliferation (Fig. 1C,D).

Accordingly, Fig. 2A shows that heme (10 μM) induces a strong ROS production after 1 h of incubation with VSMC. Corroborating recent studies that showed heme as an inductor of ROS generation via NADPHox in neutrophils, macrophages and in VSMC [7,8,19], the pretreatment of cells with DPI (10 μM), a NADPHox inhibitor [20], prevented heme-induced ROS production in A7r5 VSMC (Fig. 2A). Moreover, DPI strongly inhibited heme-induced VSMC migration (Fig. 2B) and blunted the proliferative capability of heme-treated VSMC that reached levels below the controls. (Fig. 2C), indicating that heme effects on VSMC migration and proliferation are modulated by NADPHox.

3.2. Heme activates redox-sensitive signaling pathways

Among the redox-sensitive molecules, ERK-2, a member of MAPK family, is of crucial importance to VSMC proliferation and migration [21]. In VSMC pretreated with the ERK-2 inhibitor PD98059 (10 μM), migration induced by heme was partially inhibited (Fig. 3A) and heme proliferative effect on VSMC was impaired (Fig. 3B). Supporting the data, heme induced ERK-2 phosphorylation in VSMC (Fig. 3C), an effect that was inhibited in cells pretreated with DPI (Fig. 3C).

NF-κB is another redox-sensitive pathway and a possible target of ERK-2 pathway [22]. NF-κB activation can be evaluated by its translocation to nucleus, where this factor promotes gene transcription. The heme effect on NF-κB activation was detected 1 h after VSMC stimulation and, similar to ERK-2 phosphorylation, was strongly inhibited by DPI, as evaluated by immunocytochemistry and western blotting (Suppl. Fig. 1). This effect is ERK/MAPK dependent, once PD98059 inhibited the nuclear translocation of NF-κB induced by heme (Suppl. Fig. 1A). The data suggest that NADPHox activation by heme mediates the activation of redox-sensitive signaling pathways involved in VSMC migration and proliferation.

3.3. Heme oxygenase activity down-modulates the heme effect

Heme is endogenously metabolized by HO system, generating carbon monoxide (CO), iron and biliverdin, which can be converted to bilirubin under catalytic activity of biliverdin reductase. In VSMC challenged with heme for 24 h there was no change in the expression of constitutive HO-2. On the other hand, heme induced HO-1 expression in VSMC (Suppl. Fig. 2). Corroborating this data, we observed that heme was able to induce HO-1 mRNA expression; however heme did not affect NOX4 mRNA expression (one of the major NADPHox expressed in VSMC; Suppl. Fig. 3). We discarded

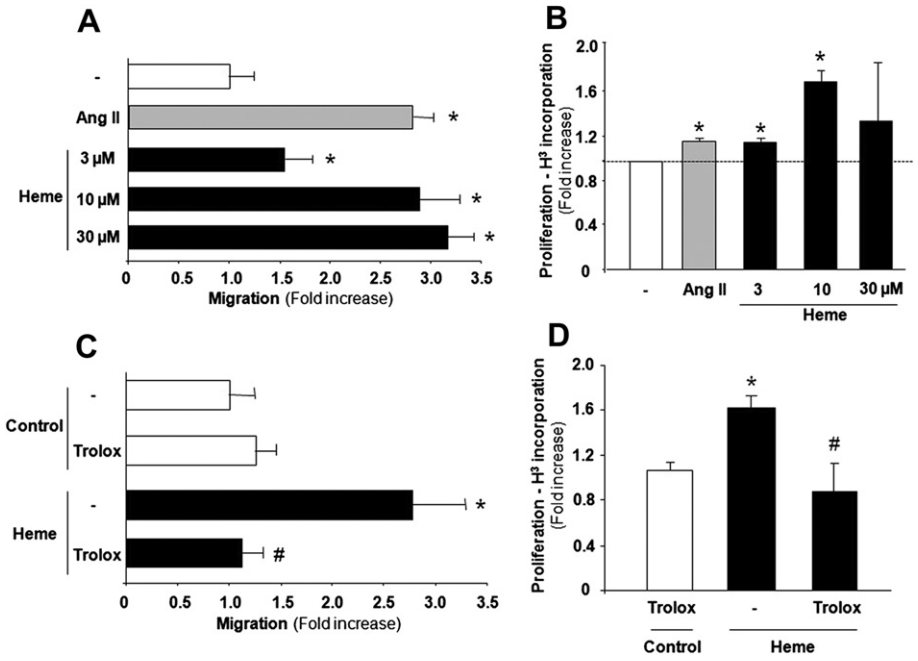

Fig. 1. VSMC migration and proliferation induced by heme are redox-dependent. (A) Migration of VSMC toward indicated concentrations of heme (3–30 μM) or Ang II (100 nM) in a Boyden chamber after 4 h incubation. (B) Proliferation of VSMC treated with heme (3–30 μM) or Ang II (100 nM) for 48 h at 37 °C, determined by thymidine incorporation assay. (C-D) VSMC were pretreated with Trolox™ (100 μM) and stimulated with heme (10 μM) for migration (C) or proliferation (D). Angiotensin II (Ang II) was used as positive control for migration and proliferation. Results are show as fold of increase in relation to cells treated with medium alone (control). The results are representative from three to five different experiments. Data are means ± SD (*$p < 0.05$ versus control; #$p < 0.05$ versus heme).

a role to NOX2, once apocynin was not able to inhibit heme effect on VSMC proliferation (data not shown).

In order to investigate the role of HO system on heme effects, VSMC were treated with the HO pan inhibitor, tin protoporphyrin IX (SnPP 3 μM) or with the selective HO-1 inhibitor, zinc protoporphyrin IX (ZnPP 10 μM). Fig. 4 shows that the inhibition of HO activity by both, SnPP (Fig. 4A) or ZnPP (Fig. 4B), potentiates the effect of heme on VSMC proliferation. Additionally, it was observed that inhibition of HO-1 by ZnPP induced a further increase in heme-induced ROS production (Fig. 4C), suggesting that activation of HO

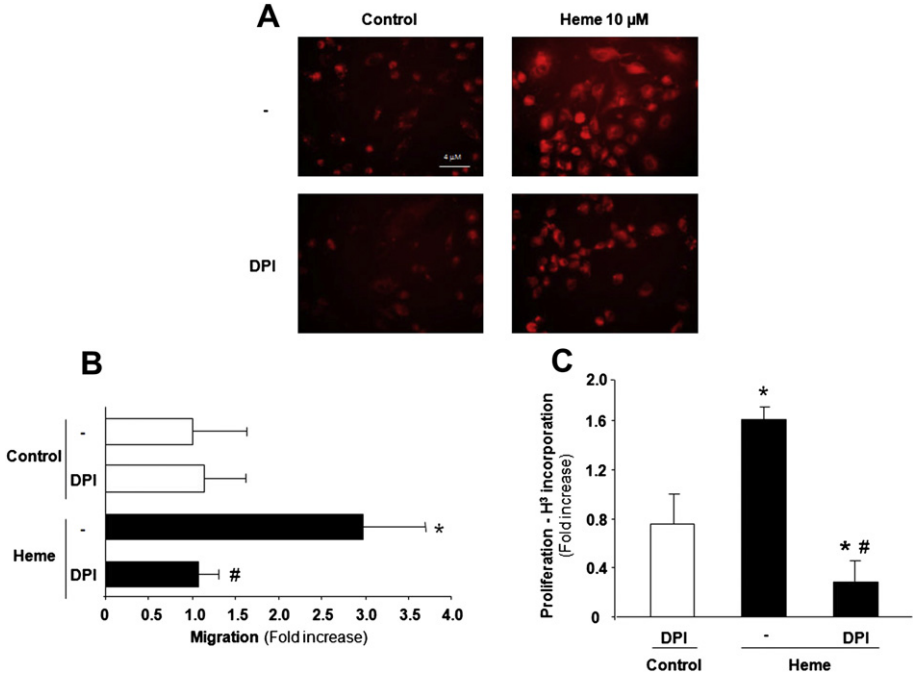

Fig. 2. Inhibition of NADPH oxidase activity impairs heme effects on VSMC. (A) Heme-induced ROS production by VSMC: Cells were incubated with or heme (10 μM/1 h/37 °C) in the absence or in the presence of a NADPHox inhibitor DPI (10 μM). ROS production was assessed by imunofluorescence using dihydrorhodamine 123 (DHR) as probe as described in Methods. The results are representative from four experiments. (B) Inhibition of NADPHox by DPI prevents heme-induced cell migration: VSMC pretreated with DPI (10 μM) were allowed to migrate to heme (10 μM) for 4 h at 37 °C in a Boyden chamber. (C) Inhibition of NADPHox by DPI prevents heme-induced cell proliferation: VSMC were incubated with or without heme (10 μM) for 48 h at 37 °C and when indicated were pretreated with DPI. Cell proliferation was determined using the thymidine incorporation assay. Results are show as fold of increase in relation to cells treated with medium alone (control). Data are means ± SD (*$p < 0.05$ versus control; #$p < 0.05$ versus heme).

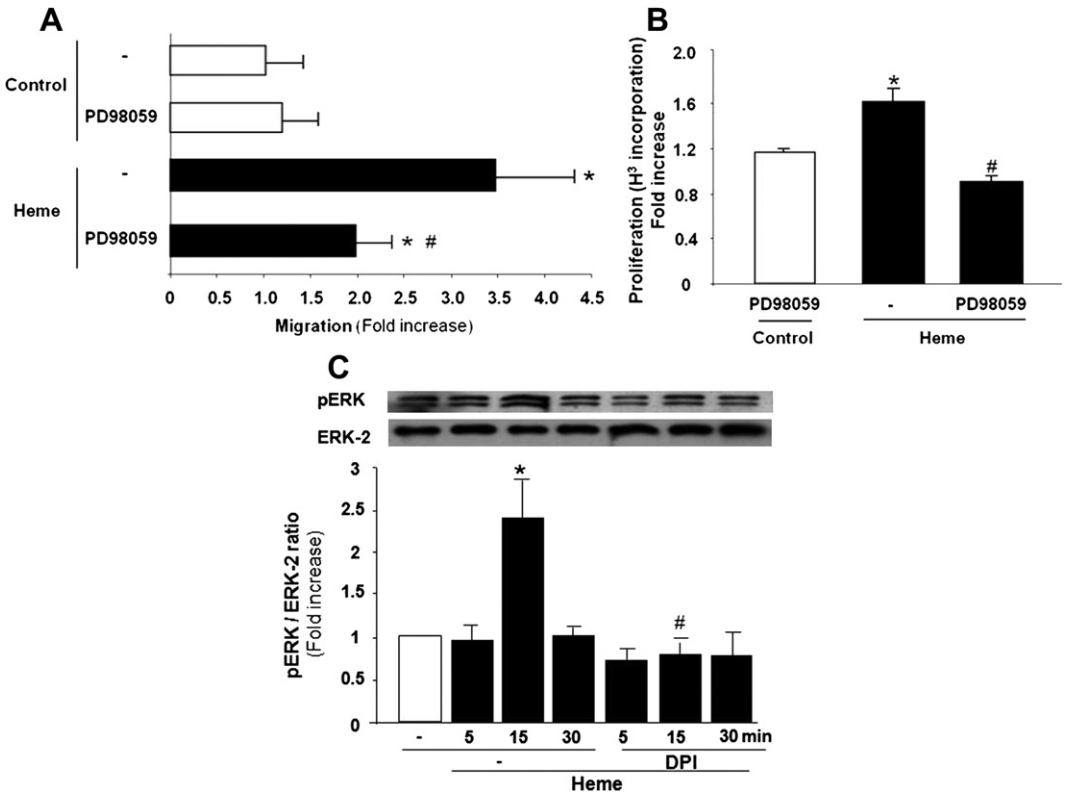

Fig. 3. Heme activates redox-sensitive signaling pathways. Inhibition of ERK activation by PD98059 (10 μM) inhibits the effect of heme (10 μM) on (A) migration and (B) proliferation of VSMC. Results are show as fold of increase in relation to cells treated with medium alone (control). Data are means ± SD (*$p < 0.05$ versus untreated group; #$p < 0.05$ versus heme). (C) Heme induced ERK phosphorylation is dependent on NADPHox activation: Cultured VSMC treated with heme (10 μM) for indicated times at 37 °C in the presence or not of DPI (10 μM). Whole-cell lysates were immunoblotted with anti-ERK polyclonal (upper inset) and with anti-ERK-2 polyclonal antibody (lower inset).

system attenuates the NADPHox-dependent activation of VSMC induced by heme.

3.4. Heme metabolites counter-regulate the heme effect

To investigate a possible counter-regulatory effect of HO metabolites on heme-induced proliferation, VSMC were treated with biliverdin (10 μM), bilirubin (10 μM) and/or with the CO donor, CO-RM (10 μM) (Fig. 5A). The metabolites had no effect on the basal neither in heme-induced VSMC proliferation. However, the potentiating effect of HO inhibitor on heme-induced proliferation was impaired when the VSMC were treated with heme metabolites (Fig. 5A). Furthermore, when both systems, NADPHox and HO, were blocked, the heme effect on VSMC proliferation was abrogated (Fig. 5B). The same was observed when the cells were treated with the antioxidant Trolox™ and SnPP (Fig. 5B), suggesting that the by-products of both systems may act together to modulate heme effect on VSMC.

4. Discussion

In some regions of the vasculature, the increase of the blood turbulence promotes the destruction of fragile or old red blood cells within the vascular compartment, allowing hemoglobin to escape to plasma. Although hemoglobin rapidly binds to serum proteins like haptoglobin, during extensive hemolysis the amount of cell-free hemoglobin exceeds the binding capacities of those hemoglobin-scavenging mechanisms, resulting in intravascular free heme release and accumulation [2].

It has been well demonstrated that heme acts as a double-edged sword. In physiological conditions, as a prostetic group of hemoproteins, heme is critically important for various biological processes. However, the free heme, released from hemoproteins by oxidative stimuli is pro-oxidant and enhances oxidative stress and inflammation [6,23]. Recent studies have shown that free heme acts as a danger signal which can induce pro-inflammatory proteins expression, ROS generation and the activation of redox-sensitive molecules in cultured cardiac resident cells [24]. ROS produced in the vasculature contribute to the pathogenesis of cardiovascular conditions, and a critical source of ROS is the vascular NADPHox. The upregulation of this enzyme brings about the oxidative stress underlying atherosclerosis and NADPHox activity has been highlighted as a potential target for pharmacological interventions in cardiovascular diseases [9,25]. Furthermore, evidences have shown that heme is a potent inductor of LDL oxidation [26] and may contribute to atherogenesis.

It is well known that heme is able to induce NADPHox in different cell types, such as neutrophil, macrophage and endothelium [6–10,14]. In this work we demonstrate that heme directly activates VSMC, promoting cell proliferation and migration, suggesting a key role of this molecule in vascular dysfunction observed during hemolytic episodes. These effects are dependent on its ability to promote ROS production through the activation of the NADPHox system. Heme-induced ROS generation triggers redox-sensitive signaling pathways, such as ERK-2 and NF-κB, which are involved in VSMC proliferation. We also investigated a possible heme effect on others MAPK and we observed that heme had no effect on p38 and JNK phosphorylation (data not shown).

On the other hand, heme is known as the main inductor of HO-1 system, which acts as potent antioxidant, protecting the vasculature, mainly attenuating the inflammatory response in the vessel

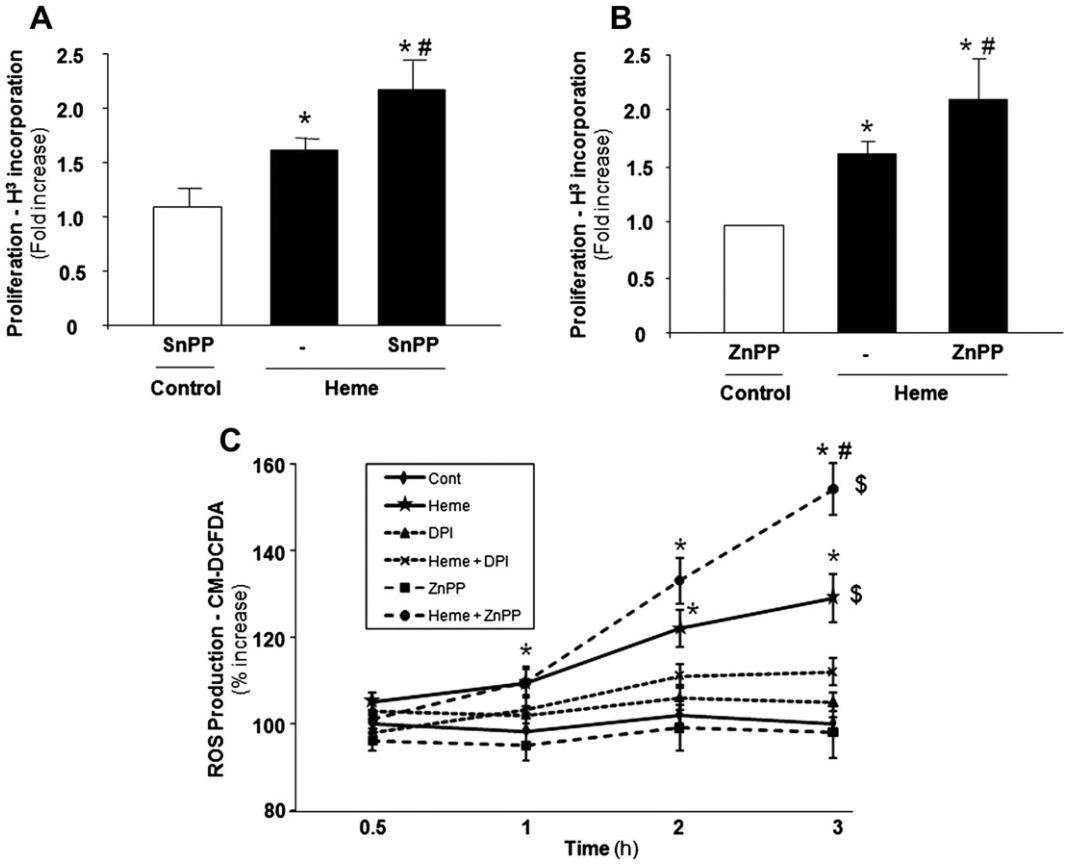

Fig. 4. Inhibitors of HO potentiate heme proliferative effect on VSMC. VSMC were incubated with heme (10 μM) for 48 h at 37 °C and when indicated were pretreated with (A) SnPP (3 μM) or (B) ZnPP (10 μM). Cell proliferation was determined using the thymidine incorporation assay. (C) HO inhibitors enhance ROS production induced by heme in VSMC: VSMC probed with CM-H_2-DCFDA were incubated for 180 min/37 °C with heme (10 μM) in the presence or not of ZnPP (10 μM) and/or DPI (10 μM). ROS production along the time was detected by fluorescence as described in Methods. The results are representative of three to five independent experiments. Data are means ± SD (t-student test in each point: $^*p < 0.05$ versus control; $\#p < 0.05$ versus heme. Two-way anova: $\$p < 0.05$ versus control).

wall [27]. Although biliverdin and CO generated by heme metabolism can be toxic at very high concentrations, recent evidence indicates that they are not toxic at physiological concentrations in normal cells, and may have important anti-oxidant and anti-inflammatory properties [16]. Supporting this idea, our data suggest that the induction of HO-1 by heme would be a key factor in the cell defense mechanism against the oxidative processes triggered by the activation of NADPHox induced by heme itself.

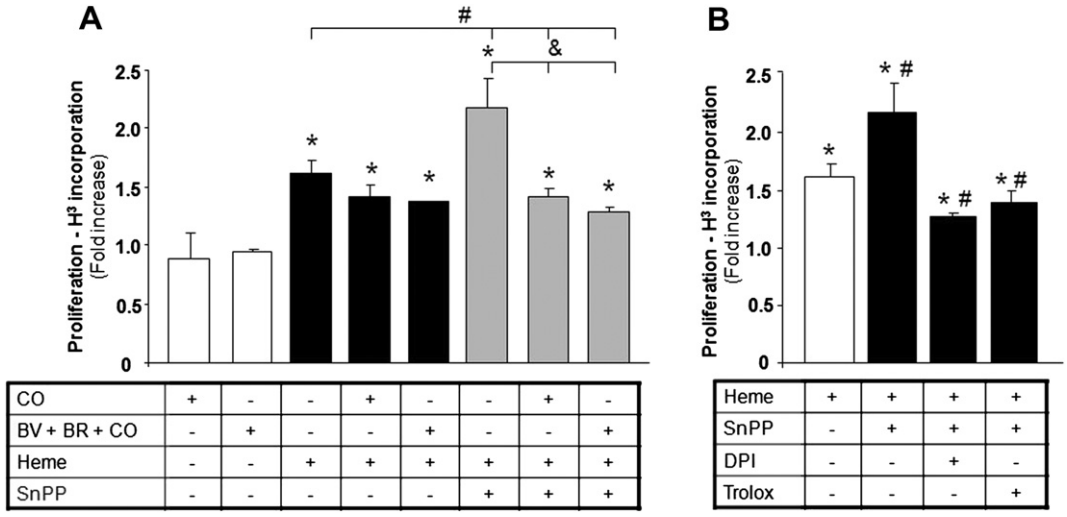

Fig. 5. The proliferative effect of heme on VSMC is modulated by NADPHox and HO activities. (A) Effect of HO metabolites on heme-induced proliferation: VSMC were pretreated with a carbon monoxide-releasing molecule (CO: 10 μM) alone, or with biliverdin (BV: 10 μM) and bilirubin (BR: 10 μM), or in the presence of SnPP (3 μM), or medium alone before incubation with heme (10 μM) for 48 h at 37 °C; and (B) Effect of both HO and NADPHox inhibition on heme-induced proliferation. Cultured VSMC were incubated with heme (10 μM) for 48 h at 37 °C and when indicated were pretreated with SnPP (3 μM), DPI (10 μM) or Trolox™ (100 μM). The results are representative of three to five independent experiments. Data are means ± SD ($^*p < 0.05$ versus control; $\#p < 0.05$ versus heme; $\&p < 0.05$ versus heme + SnPP).

In our experimental model, the challenge of VSMC with heme induces HO-1, without affecting the HO-2 expression. The inhibition of HO activity with a pan-inhibitor SnPP, or with ZnPP, an HO-1 selective inhibitor, potentiates the proliferative effect of heme on VSMC. This effect seems to be a consequence of an increase in heme bioavailability (Suppl. Fig. 4), which leads to increased ROS production, and an impairment in the generation of HO-derived antioxidant products. Accordingly, as shown in Fig. 5, the addition of CO/biliverdin and bilirubin impairs the potentiation of HO inhibitor on heme proliferative effect. Those heme metabolites, due to their antioxidant properties [28], are described to inhibit MAPK pathway in VSMC [19,29,30], which confers an important anti-proliferative role for these molecules. Furthermore, heme metabolites [30,31] were described to induce cell cycle arrest in G0 phase. Corroborating the data, we have also observed that when NADPHox activity was inhibited, heme induces cell cycle arrest in G0 phase, probably via heme metabolites. On the other hand, the inhibition of HO accelerates G2 phase induction by heme, indicating an anti-proliferative effect of HO by-products (Suppl. Fig. 5). These results were confirmed by MTT assay, showing that the proliferative effect of heme on VSMC was potentiated by HO inhibition and prevented in the presence of a NADPHox inhibitor (Suppl. Fig. 6).

A balance between the activity of NADPHox and HO systems modulating different cell functions has been frequently reported. HO-1 induction has potent protective actions against ROS-induced oxidative damage both in vitro and *in vivo* [27,32]. A recent report has demonstrated a link between ROS-generated from NADPHox and HO-1 activation during mesenteric arteries remodeling [33]. Additionally, Bellner & colleagues [15] observed that HO-2 deletion induced NADPHox activation, ROS increase and NF-κB activation in endothelial cells.

Interestingly, in our experimental model, heme (10 μM) effect on NADPHox overcomes HO system activity, culminating on VSMC proliferation. Our data contrast with others by Chang and colleagues demonstrating that the treatment of VSMC with heme at 5 μM for 7–21 days inhibited proliferation, via HO-1 [34]. On the same direction, Hyvelin et al., showed *in vivo* that HO-1 induced by heme (30 uM – plasma concentration), prevented the in-stent stenosis in rat or rabbit after 28 days [35]. So, it is conceivable to assume that the prolonged treatment with heme promotes a long lasting upregulation of HO system, which overcomes NADPHox activation, leading to inhibition of VSMC proliferation. Additionally, the short half-life of heme (Suppl. Fig. 4) may explain the rapid effect on NADPHox and the longtime of the anti-proliferative effect by HO system (HO-1 and by heme metabolites).

It is well-established that HO system down-modulates VSMC proliferation and that heme has pro-oxidant properties, however we are now showing, consistently, for the first time that the treatment with heme triggers the VSMC proliferation, which is dependent on ROS derived from the NADPHox activation and associated to the activation of ROS-sensitive signaling pathways. Moreover, we show that the proliferative effect on VSMC activated via the pro-oxidant properties of heme is endogenously regulated through the balance between the activity of NADPHox complex and HO system, particularly HO-1, which diminishes heme bioavailability as well as generates antioxidant/anti-proliferative metabolites (Suppl. Fig. 7). Thus, concomitantly, endogenous HO-derived by-products are acting to minimize the pro-oxidant effect of heme, and the inhibition of HO activity leads to a potentiation of heme proliferative effect.

A better knowledge of the underlying mechanisms involved in heme effects on NADPHox and HO systems may lead to the development of new strategies for the treatment of vascular disorders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.043.

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