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Heme Oxygenase-1 Inhibition Prevents Intimal Hyperplasia Enhancing Nitric Oxide-Dependent Apoptosis of Vascular Smooth Muscle Cells

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Received December 20, 2010; accepted April 16, 2011; published online May 12, 2011

Heme oxygenase-1 (HO-1, encoded by the *HMOX1* gene) and inducible nitric oxide synthase (iNOS) have been implicated in vascular disease; however the role of these genes remains unclear. Therefore, we studied the mechanism by which iNOS-derived nitric oxide (NO) affects the intimal hyperplasia (IH) formation in relation to HO-1. We show, in a model of balloon injury in rats, that the suppression of vascular smooth muscle cells (VSMC) proliferation by NO required HO-1, while induction of apoptosis of the VSMC by NO does not involve HO-1. To better clarify the molecular mechanism of this finding, we used *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC exposed to NO. In *Hmox1*$^{+/+}$ VSMC, NO is antiproliferative (up to 34% inhibition) and it is associated to an increase of apoptosis (up to 35%) due to a decrease of X-linked inhibitor of apoptosis protein (XIAP) expression level and to the activation of caspase-3. In the absence of HO-1 (*Hmox1*$^{-/-}$ VSMC) apoptosis was significantly greater (69% $p<0.01$ *vs. Hmox1*$^{+/+}$ VSMC) demonstrating that HO-1 attenuated the pro-apoptotic effect of NO on VSMC. In the context of IH, the pro-apoptotic effect of NO on VSMC is increased in the absence of HO-1 and exerts therapeutic effects with a significant reduction in IH.

Key words nitric oxide; heme oxygenase-1; proliferation; apoptosis; intimal hyperplasia

Vascular injury causes endothelial cell damage resulting in the recruitment of bone marrow stem cells and in the activation of medial vascular smooth muscle cells (VSMC), which proliferate, migrate to the intima and increase synthesis of extracellular matrix, leading to stenosis.[1—3] Impairment of endothelial cells results in an increase in inducible nitric oxide synthase (iNOS) expression and activity. Elevated levels of nitric oxide (NO), synthesized by iNOS are believed to contribute to tissue damage by inducing an inflammatory response.[4] NO, formed from L-arginine, is in fact involved in a variety of vascular, physiological and pathological processes.[5] Recently, it has been shown that cells able to produce NO possess also cytoprotective enzymes to limit the potential damage of NO including heme oxygenase-1 (HO-1).[6,7] HO-1 inhibits the growth of VSMC *in vitro* and *in vivo*, causes G_1/S growth arrest and up-regulation of $p21^{Cip1}$, consistent with the idea that HO-1 participates in the regulation of VSMC proliferation in injured vessels.[8,9] The HO-1 gene transferred to balloon-injured rat carotid artery induces vascular HO-1 protein expression and strongly attenuates neointima formation in response to injury.[10] HO-1 provides protection against inflammation and oxidant-mediated injury preventing apoptotic cell death and regulating cell proliferation as shown by several previous studies.[11—13] It has also been proposed that HO-1 activity may serve under certain circumstances to modulate NO production. HO-1 induction is likely involved in some of the actions of NO, such as the ability to react non-specifically with many cellular components causing deleterious effects.[14] Chemical inhibition of HO-1 activity by zinc protoporphyrin-IX in VSMC results in increased NO production, suggesting that HO-1 may exert an inhibitory effect on NOS.[15] Likewise, both increased HO-1 activity and exogenously administered carbon monoxide (CO) have been shown to inhibit NOS and thereby suppress NO generation.[14]

Although it has been reported that there are metabolic and functional links between these inducible enzymes through the action of their gaseous products, CO and NO, the relationship between iNOS-derived NO and HO-1 remains to be clarified.[16]

Therefore in the present study we wanted to investigate the role of HO-1 and iNOS in the prevention of intimal hyperplasia (IH) formation in a model of balloon-injury (BI) in rats and *in vitro* by using VSMC. Moreover, we attempted to elucidate the molecular mechanism by which these molecules control proliferation and/or apoptosis of VSMC in injured vessels. The results show that in the absence of HO-1, iNOS expression and activity are significantly higher in *Hmox1*$^{-/-}$ compared to *Hmox1*$^{+/+}$ resulting in VSMC apoptosis.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats weighing 350—400 g (15—20 weeks old) were purchased from Harlan Italy. Animals were fed with standard chow and water *ad libitum* and housed under controlled conditions of temperature (24±1 °C), humidity and light (12 h light/dark cycles). Experiments involving animals were carried out according to a protocol approved by the animal care committee of the Minister of Public Health, and in accordance with guidelines from the European Union and NIH guide for use and care of laboratory animals.

Treatments Rats were randomly assigned to 4 experi-

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mental groups ($n=8$ each). Rats were treated for 24 h before the surgical procedure with L-arginine hydrochloride (2.25%, Sigma-Aldrich, U.S.A.), in the drinking water, alone or in association with a single dose of N^{ω}-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg of body weight intraperitoneally (i.p.) Sigma-Aldrich, U.S.A.), as NOS inhibitor, or tin protoporphyrin-IX (SnPP-IX; 50 μmol/kg of body weight i.p. Frontier Scientific Inc., U.S.A.), as HO-1 inhibitor.[17—19] SnPP-IX was dissolved in 0.1 N NaOH, neutralized with HCl and diluted to the final volume with NaCl 0.9%. L-NAME was dissolved in normal sterile saline solution. Control was animals subjected to BI. Uninjured contralateral (sham) carotid arteries of each animal were used as internal control.

Surgical Model Rats were initially anesthetized with 5% isoflurane (Abbott Laboratories, U.S.A.) and maintained at 3% in a 30% : 70% O_2/N_2O mixture throughout the procedure. Balloon-denudation was performed in the left common carotid artery as previously described.[20] After 14 d from the surgical procedure rats were humanly sacrificed and both carotids (left and right) were harvested.

Morphometric Analysis A section of carotid artery corresponding to the location of the lesion was excised and fixed with 4% paraformaldehyde, embedded in paraffin and cut into 3 μm slices. Ten serial sections for each carotid artery were selected and used for hematoxylin/eosin (H&E) staining and for morphometric analyses. Quantitative morphometric analyses of IH were performed using a video camera at 10× magnification (ScanScope) and computerized digital image analysis (ImageScope, Aperio). To better visualize the vascular damage the sections of the carotids were also photographed with a light microscopy at 40× and examined in autofluorescence (Axioskope 40, Zeiss).

Immunohistochemistry Tissue sections were dewaxed and antigen was retrieved by 10 mM buffer citrate pH 6.0 at 98 °C for 25 min (Dako). Endogenous peroxidase activity was blocked and sections were incubated with nonimmune serum for 30 min and then incubated with the primary antibody for 1 h. The following primary antibodies were used: mouse monoclonal anti-Ki-67, against actively dividing nuclei (MM1, Vector Laboratories, 1 : 100); rabbit polyclonal anti-cleaved caspase-3 (Asp175, Cell Signaling Technology, 1 : 50); rabbit polyclonal anti-iNOS (N20, Santa Cruz Biotechnology, U.S.A., 1 : 100); rabbit polyclonal anti-HO-1 (SPA-895, StressGen Biotechnology, U.S.A., 1 : 500). After washing, slides were incubated for 30 min with the appropriate biotin-conjugated secondary antibody. Biotinylated horse anti-mouse immunoglobulin G (IgG) rat adsorbed (Vector Laboratories, 1 : 200) was used to detect the stains with antibody against Ki-67 and goat anti-rabbit IgG to detect the stains of cleaved caspase-3, iNOS and HO-1 (Vectastain ABC Kit, Vector Laboratories, 1 : 200). Treatment with the secondary antibodies was followed by incubation with avidin–biotin–peroxidase complex for 30 min. Immunostaining were visualized by using 3′3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, U.S.A.). Sections were counterstained with Mayer's hematoxylin solution (Dako), then rinsed with distilled water, dehydrated and mounted (Vector Laboratories). No-specific staining was detected by omission of the primary antibodies. All slides were photographed using a video camera at 40× magnification. The percentage DAB-positive cells (for HO-1 or Ki-67) was determined using computerized digital image analysis (ImageScope, Aperio). Results were expressed as the ratio between brown DAB positive cells and total cells per field. The percentage of DAB-positive area (for cleaved caspase-3 or iNOS stained sections) was evaluated using ImageScope software, Aperio.

Vascular Smooth Muscle Cells Culture Murine *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMCs[21—23] were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomicyn (Gibco, Invitrogen). The cells at the seventh to fifteenth passage were used for proliferation and apoptosis assays and Western blot analysis.

Cells Treatments and Western Blot Analysis Cells were grown in DMEM supplemented with 10% of FBS to reach the confluence, and then were serum starved in DMEM 0.5% FBS and treated in different way. After 24 h cells were treated with interleukin-1β (IL-1β; 10 ng/ml, Peprotech) alone or in association with L-NAME (1 mmol/l, Sigma-Aldrich) for the time points indicated.[24,25] Untreated cells were used as a control. Alternatively, cells were treated with different concentration of (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP; 100, 300, 500, 1000, 1200 μmol/l, Calbiochem) for 24 h.[26,27] The cells cultured in DMEM alone or treated with H_2O_2 for 4 h were used as controls of the experiment. Cells were homogenized in lysis buffer [50 mmol/l Tris HCl (pH 7.4), 250 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 5% glycerol and 2% protein inhibitor cocktail (PIC; Sigma-Aldrich)]. Equal amounts of protein determined by Bradford dye (Bio-Rad Laboratories) were separated *via* SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL, Amersham). The membranes were blocked with 5% non-fat dry milk in TBST for 1 h then incubated with the appropriate dilution of antibody for 1 h at room temperature or 16 h at 4 °C with agitation. Following the incubation with HRP-labelled secondary antibodies, blots were washed and reactivity was detected using enhanced chemioluminescence (ECL, Amersham). Blots were exposed to hyperfilm (Amersham). Band intensities were quantified with densitometric scanner and values were normalized to β-actin signals. Antibodies included anti-HO-1 (SPA-895, StressGen Biotechnology), anti-iNOS (Cayman), anti-cleaved Caspase-3 (Asp175, Cell Signaling Technology), anti-ILP (N18, Santa Cruz Biotechnology) and anti-β-actin (AC-15, Sigma-Aldrich). The antibodies were used at the dilution suggested by the manufacturer. Each blot is representative of three independent experiments.

Bromodeoxyuridine (BrdU) Cell Proliferation Assay The rate of replication of *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMCs was determined by proliferation assay using BrdU dye (Cell proliferation enzyme-linked immunosorbent assay (ELISA), BrdU colorimetric, Roche). Cells were plated in quadruplicate in the same 96 well plates. Once the cells reached subconfluence, they were treated with different concentration of SNAP (500, 800, 1000, 1200 μmol/l) for 24 h. After 2 h, BrdU was added to the medium of cells with or without NO donor's stimulation. Plates were processed according to the manufacturer's recommendation. Absorbance was measured at 450 nm. Cell proliferation was expressed as percentage of BrdU-positive cells relative to untreated control, arbitrarily

representing a value of 100%. The mean and the standard deviation of the absorbance value for quadruplicate wells were calculated.

Morphological Assessment of Apoptosis and Cell Death VSMC *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ cell 80% confluent in 6 well plates were serum starved in DMEM 0.5% FBS and exposed to different concentration of SNAP (500, 800, 1000, 1200 μmol/l) for 24 h. At the different time points cells were stained with Hoechst 33342 (5 μg/ml, Invitrogen) and nuclear condensation and DNA fragmentation were visualized using an inverted phase fluorescence microscope (20× magnification, Nikon Eclipse TE 2000-S). The number of dead cells was determined using the trypan blue–exclusion test. Only cells that excluded dye were considered as live cells. The percentage of dead cells at each time point was calculated dividing the number of non-viable blue cells by the number of total cells. Results were indicative of 3 independent experiments.

Measurement of Nitrite Concentration in Rat Plasma and in VSMC Culture Media The concentration of NO_x (total nitrite) was measured in samples of plasma and in culture medium using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.). This assay converts NO_3^- to NO_2^- with nitrate reductase, and total NO is measured as total nitrite using the Griess reagent.[28] Blood samples were collected during the surgical procedure, just before the balloon injury, and plasma was separated by centrifugation, immediately frozen and stored at −80 °C until assayed. Plasma was thawed and filtered through Microcon YM-30 Centrifugal Filters (Millipore Corp.), to remove any background absorbance attributable to hemoglobin in the plasma. Culture media were incubated with Griess at room temperature. The absorbance of plasma and culture medium samples were measured and the NO_x concentration was determined by using a calibration curve of sodium nitrite. The *in vitro* results were indicative of 3 independent experiments.

Statistics Results were indicated as mean±standard error of the mean (S.E.M.) or mean±standard deviation (S.D.). Statistical analysis was performed using SPSS 17 software. Student's *t*-test was used to compare data between two treatment groups. Differences between more than two experimental groups were determined with one-way analysis of variance (ANOVA), and when significant differences among groups were found, a *post hoc* analysis (Tukey test) was used. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Pre-treatment with L-Arginine Alone or in Combination with SnPP-IX Prevents the Development of IH in an *in Vivo* Model of Balloon-Injury in Rat To characterize neointimal formation we performed morphometric analyses on hematoxilin and eosin (H&E) stained sections of rat carotid arteries 14 d after injury (Fig. 1). Intimal hyperplasia was observed in the carotid arteries subjected to BI (group I) and not in sham carotids (C) as expected. A single oral dose of L-arginine alone (group II) or in combination with SnPP-IX (group IV) significantly suppressed IH formation at the site of the injury. Treatment with L-arginine (group II) resulted in a significant decrease in intima/media (I/M) thickness and I/M area ratio compared to BI alone (group I) (1.48±0.06 *vs.* 2.11±0.08; $p \leq 0.01$ and 0.86±0.02 *vs.* 1.26±0.07; $p \leq 0.01$, respectively, Figs. 1b, c). Co-treatment with L-arginine and SnPP-IX (group IV) resulted in a significant reduction of I/M thickness ratio compared to group II (1.17±0.06 *vs.* 1.48±0.06; $p \leq 0.05$, Fig. 1b) and a reduction of I/M area ratio compared to group II (0.66±0.02 *vs.* 0.86±0.02, Fig. 1c).

Significant reduction in the degree of stenosis resulted in group II compared to group I (32.25±1.16 *vs.* 44.35±1.10; $p \leq 0.01$). The co-treatment L-arginine and SnPP-IX (group IV) further reduced the degree of stenosis compared to group II (29.72±0.66 *vs.* 32.25±1.16). Inhibition of NOS by L-NAME (group III) abrogated the protective effect of L-arginine/NO resulting in a significant increase of I/M thickness ratio, I/M area ratio and the degree of stenosis compared to group II (2.24±0.08 *vs.* 1.48±0.06, $p \leq 0.01$; 1.32±0.08 *vs.* 0.86±0.02, $p \leq 0.01$ and 39.23±1.36 *vs.* 32.25±1.16, $p \leq 0.01$).

Similar results were obtained with long-term treatments, starting from 5 d before BI and continued for fourteen days after (data not shown).

Prevention of IH Development, Following SnPP-IX Treatment, Is Related to the Balance of Apoptosis *vs.* Proliferation of VSMC To understand the mechanism by which inhibition of HO-1 and iNOS expression could prevent neointimal formation in the injured carotids, we analyzed whether the expression of these inducible enzymes was related to the modulation of VSMC proliferation and apoptosis (Fig. 2a). Proliferative index was calculated by dividing the number of Ki-67-positive nuclei by the total number of nuclei. As previously shown[16] HO-1 and iNOS expression is induced in injured vessels (group I) compared to the contralateral uninjured artery (Figs. 2a—c). Injured carotids from rats treated with L-arginine (group II), which showed a significant reduction in IH ($p<0.05$), was associated with modest elevations in iNOS protein expression compared to group I (25.6±5.6% *vs.* 17.0±1.3%; percentage of positive area; Figs. 2a, c). The co-administration of L-arginine with L-NAME as inhibitor of NOS activity (group III) led to an increment of IH and to a significant increase of iNOS protein expression compared to group II (50.7±1.2 *vs.* 25.6±5.6; percentage of positive area, $p<0.01$; Figs. 2a, c) and to group IV (50.7±1.2% *vs.* 31.1±0.5%, $p<0.05$; Figs. 2a, c). In contrast, the deficiency in HO-1 activity due to SnPP-IX treatment, in rats co-treated with L-arginine, (group IV) determined an important reduction of IH and an up-regulation of HO-1 positive cells compared to group II (94.2±3.1% *vs.* 82.3±2.8%; Figs. 2a, b). Balloon-injured vessels (group I) showed an increase in caspase-3 activation (8.0±0.01 percentage of positive area; Figs. 2a, e) and Ki-67 (9.7±0.5%; $p<0.01$; Figs. 2a, d) expressing cells compared to uninjured artery (5.9±0.2% and 2.7±0.6% respectively; Figs. 2a, d, e). Carotids from animals receiving L-arginine (group II) displayed an increase in apoptosis (11.1±0.6%; Figs. 2a, e) compared to group I. Our results also demonstrated that NOS inhibition with L-NAME significantly increased apoptosis (40.8±6.8%, $p<0.01$; Figs. 2a, e) and VSMC proliferation (20.8±0.7%, $p<0.01$; Figs. 2a, d) compared to group II (11.1±0.6% and 16±0.9%; Figs. 2a, d, e). The co-treatment

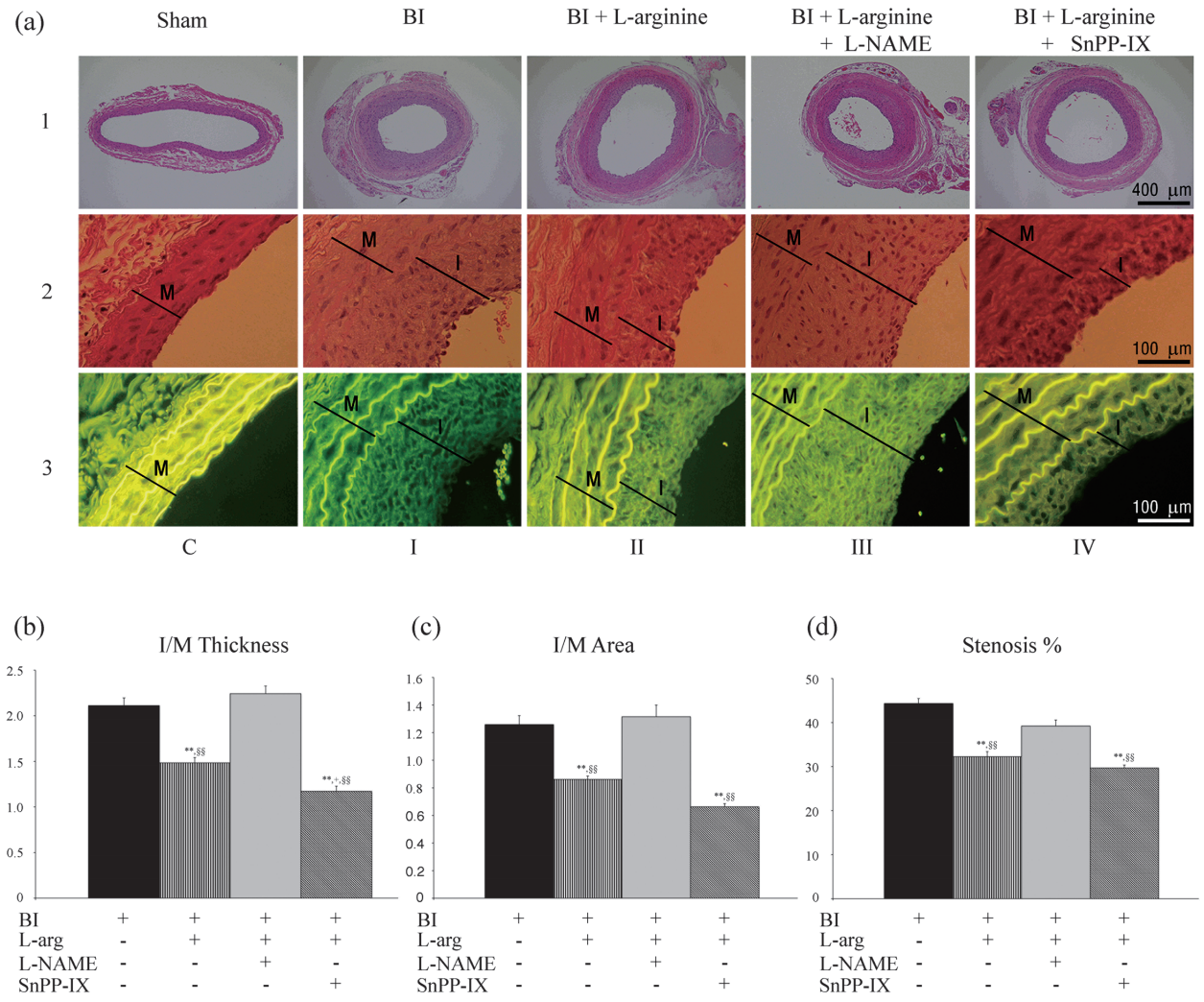

Fig. 1. Co-treatment with L-Arginine and SnPP-IX Prevents the IH in Balloon-Injured Rats

(a) Representative cross-sections of morphometric analysis of carotid arteries 14 d after BI. C) Uninjured carotid artery (sham); I) carotid from rat subjected to BI without L-arginine treatment or II) treated with L-arginine for 24 h before BI; III) carotid from rat subjected to BI and treated with L-arginine in association with a single dose of L-NAME 24 h before BI; IV) carotid from rat subjected to BI and treated with L-arginine in combination with a single dose of SnPP-IX 24 h before BI. Sections were stained with H&E, 1) light microscopy 10× magnification; 2) light microscopy 40× magnification; 3) autofluorescence 40× magnification. (b, d) Histograms illustrating: (b) I/M thickness, (c) I/M area or (d) stenosis % determined on carotid arteries from animals of groups I—IV. Data are expressed as mean±S.E.M.%. $^{**}p<0.01$ *vs.* group I $^{\S\S}p<0.01$ *vs.* group III; $^{+}p<0.05$ *vs.* group II. M=media; I=intima.

Table 1. Effects of the Delivery of L-Arginine Alone or in Combination with L-NAME or SnPP-IX on Nitrite Concentrations in Plasma of Rats

Groups	BI	L-Arginine	L-NAME	SnPP-IX	NO_x (μM)
Control (n=8)	−	−	−	−	undet.
BI (n=8)	+	−	−	−	2.24±0.27
L-Arginine (n=8)	+	+	−	−	3.10±0.25
L-NAME (n=8)	+	+	+	−	undet.
SnPP-IX (n=8)	+	+	−	+	6.06±0.35$^{a,b)}$

Data are expressed as mean concentration of nitrites (NO_x)±S.E.M. NO_x: total concentration of nitrates and nitrites; Control: untreated and uninjured rats; undet.: undetectable, <1 μM. *a*) $p<0.01$ *vs.* group BI. *b*) $p<0.01$ *vs.* group L-arginine.

with SnPP-IX and L-arginine resulted in a remarkable induction of the apoptotic cell death (55.9±4.2%, $p<0.01$; Figs. 2a, e) and in an increase in VSMC proliferation (28.6±0.8%, $p<0.01$; Figs. 2a, d) compared to group II indicating that when HO-1 activity is inhibited the anti-proliferative effect on VSMC is lost.

The Inhibition of HO-1 by SnPP-IX Leads to a Significant Increase of Total Plasma NO Concentrations The administration of L-arginine increased the NO_x concentration in the plasma of rats compared to untreated and uninjured group (Table 1; 3.10±0.25 μM *vs.* undetectable) and BI group (2.24±0.27 μM). Plasma NO_x levels were approximately doubled in rats co-treated with L-arginine and the inhibitor of HO-1, SnPP-IX, compared to L-arginine group (Table 1; 6.06±0.35 μM *vs.* 3.10±0.25 μM, $p<0.01$) and to BI group (2.24±0.27 μM, $p<0.01$). Conversely the L-NAME/L-arginine co-treatment prevented plasma nitrite production (undetectable) in rats subjected to balloon injury. Since the inhibition of HO-1 led to an increase of NO production in injured vessel and to a decrease of intimal hyperplasia, the *in vitro* experiment was performed to further analyze the role of HO-1 in the production of NO.

HO-1 Modulates IL-1β-Induced iNOS Expression and Activity in VSMC The response to vascular injury involves many different processes including the up-regulation and release of pro-inflammatory cytokines such as interleukin (IL)-1β and the activation of inducible genes such as

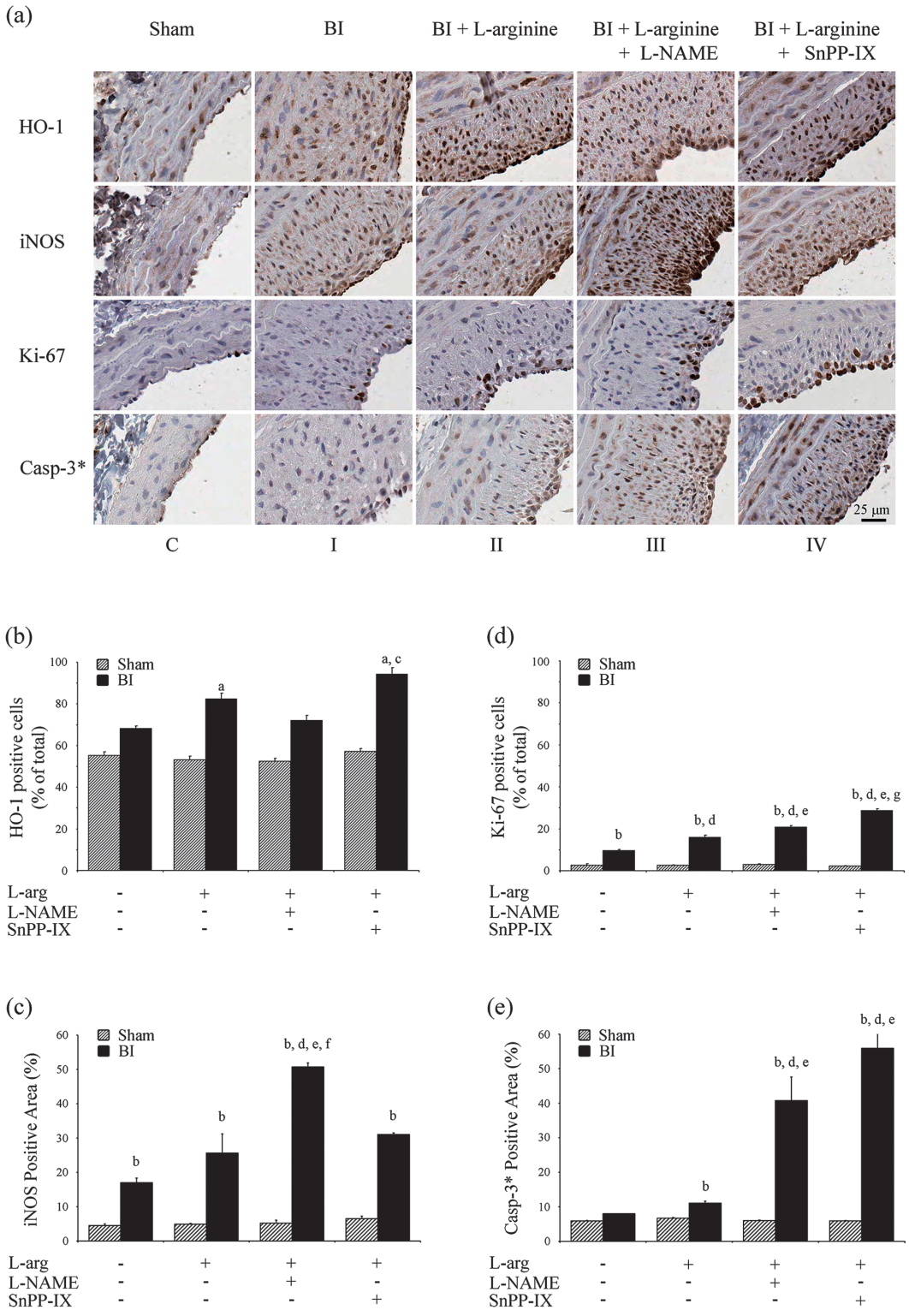

Fig. 2. SnPP-IX Prevented IH after BI Increasing the VSMC Apoptosis

(a) Representative cross-sections of immunohistochemical analysis of rat carotid arteries 14 d after BI treated as described in Fig. 1. Samples were stained with primary antibodies specific for HO-1, iNOS, Ki-67 and casp-3* (40× magnification). Proliferative index was calculated by dividing the number of Ki-67-positive nuclei by the total number of nuclei. (b, d) Evaluation of HO-1-positive cells (b) and Ki-67-positive nuclei (d) in the contralateral carotid arteries (hatched bars) and in the BI carotid arteries (closed bars). The histograms represent the average number±S.E.M. of HO-1- and Ki-67-positive cells/total cells. (c, e) Evaluation of iNOS (c) and casp-3* (e) expression in the contralateral carotid arteries (hatched bars) and in the BI carotid arteries (closed bars). The histograms represent the percentage of positive area±S.E.M. of iNOS and casp-3* per group. [a] $p<0.05$ *vs.* sham carotids; [b] $p<0.01$ *vs.* sham carotids; [c] $p<0.05$ *vs.* group I; [d] $p<0.01$ *vs.* group I; [e] $p<0.01$ significant *vs.* group II; [f] $p<0.05$ significant *vs.* group IV; [g] $p<0.01$ significant *vs.* group III. Casp-3*=cleaved caspase-3.

HO-1 and iNOS.[29—31] In an attempt to mirror the *in vivo* inflammation following injury we treated *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC with IL-1β *in vitro* (Fig. 3). *Hmox1*$^{+/+}$ VSMC (Fig. 3a) showed an increase in HO-1 protein expression between 8 h ($p<0.01$ *vs.* Control) and 16 h ($p<0.01$ *vs.* Control) followed by a decrease between 24 h and 48 h after

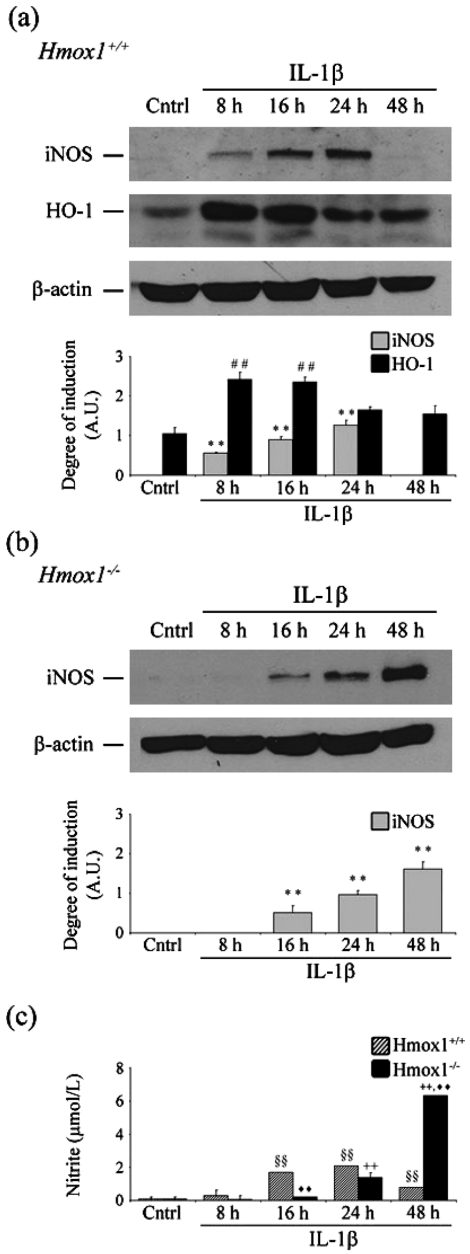

Fig. 3. HO-1 Can Control IL-1β-Induced iNOS Expression and Activity in VSMC

(a) *Hmox1*$^{+/+}$ and (b) *Hmox1*$^{-/-}$ VSMC were treated with IL-1β (10 ng/ml) for the indicated time points. Cells lysates were harvested and iNOS and HO-1 were evaluated by Western blotting using specific antibodies. β-Actin antibody was used to demonstrate equal protein loading. $^{**}p<0.01$ *vs.* Cntrl, $^{\#\#}p<0.05$ *vs.* Cntrl. (c) iNOS activity was assessed by measurement of nitrite concentration in culture medium using Griess reagent. $^{\S\S,++}p<0.01$ *vs.* Cntrl; $\blacklozenge\blacklozenge p<0.01$ *vs.* *Hmox1*$^{+/+}$ VSMC. Data represent the mean±S.D. of 3 independent experiments. A.U.=arbitrary units.

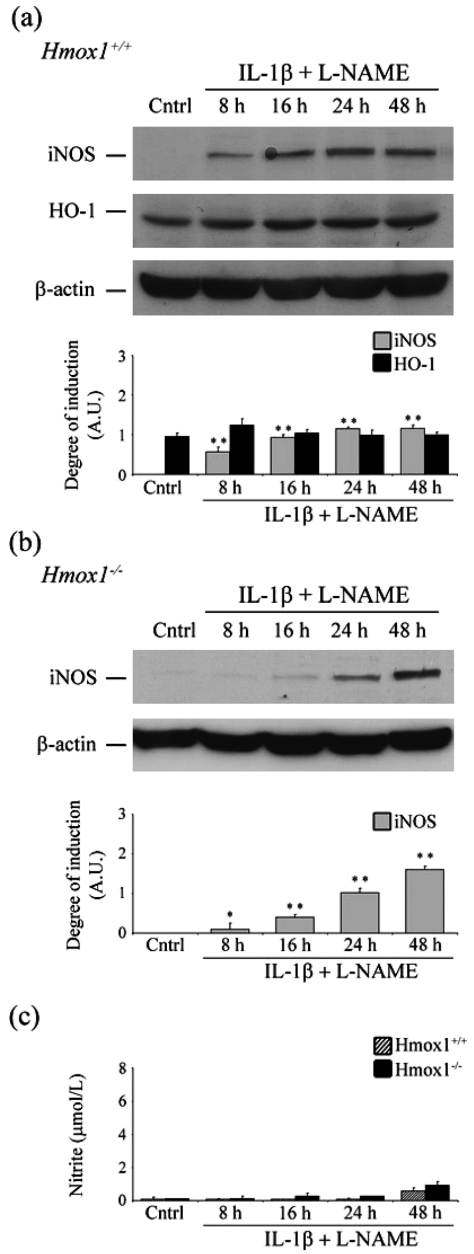

Fig. 4. Inhibitor of NO-Releasing Significant Reduced HO-1 Expression

(a) *Hmox1*$^{+/+}$ and (b) *Hmox1*$^{-/-}$ VSMC were treated with IL-1β (10 ng/ml) in combination with L-NAME (1 mmol/l) for the indicated time points. In the control group cells were incubated with the medium alone. Cells lysates were harvested and iNOS and HO-1 were evaluated by Western blotting using specific antibodies. β-Actin antibody was used to demonstrate equal protein loading. $^{*}p<0.05$ *vs.* Cntrl; $^{**}p<0.01$ *vs.* Cntrl. (c) iNOS activity was assessed by measurement of nitrite concentration in culture medium using Griess reagent. Data represent the mean±S.D. of 3 independent experiments. A.U.=arbitrary units.

treatment with IL-1β. The pro-inflammatory stimulus induced also iNOS enzyme expression (Figs. 3a, b) and NO production (Fig. 3c) in both cell lines. However in *Hmox1*$^{-/-}$ cells exposed to IL-1β the iNOS expression and activity was significantly greater than in *Hmox1*$^{+/+}$ cells ($p<0.01$ at 48 h after induction) with a different time-course of induction. In *Hmox1*$^{+/+}$ VSMC (Fig. 3a) the up-regulation of iNOS expression started after 8 h of treatment ($p<0.01$ *vs.* Control), increased through 24 h ($p<0.01$ *vs.* Control) and disappeared at 48 h whereas in *Hmox1*$^{-/-}$ VSMC the induction of iNOS is delayed, starting at 16 h (Fig. 3b, $p<0.01$ *vs.* *Hmox1*$^{+/+}$) and increasing from 24 to 48 h. Up-regulation of iNOS protein was accompanied by an increased iNOS activity assessed by measurement of nitrite concentration in culture medium by the Griess reaction (Fig. 3c). These results showed that in absence of HO-1, iNOS expression ($p<0.01$) and activity ($p<0.01$) were significantly higher than *Hmox1*$^{+/+}$ after 48 h of IL-1β treatments.

We also examined the relationships between HO-1 and iNOS during the inflammatory stimulus by abating the NO contribution. In *Hmox1*$^{+/+}$ VSMC co-stimulation with IL-1β and L-NAME, an inhibitor of NO production, (Fig. 4a) abol-

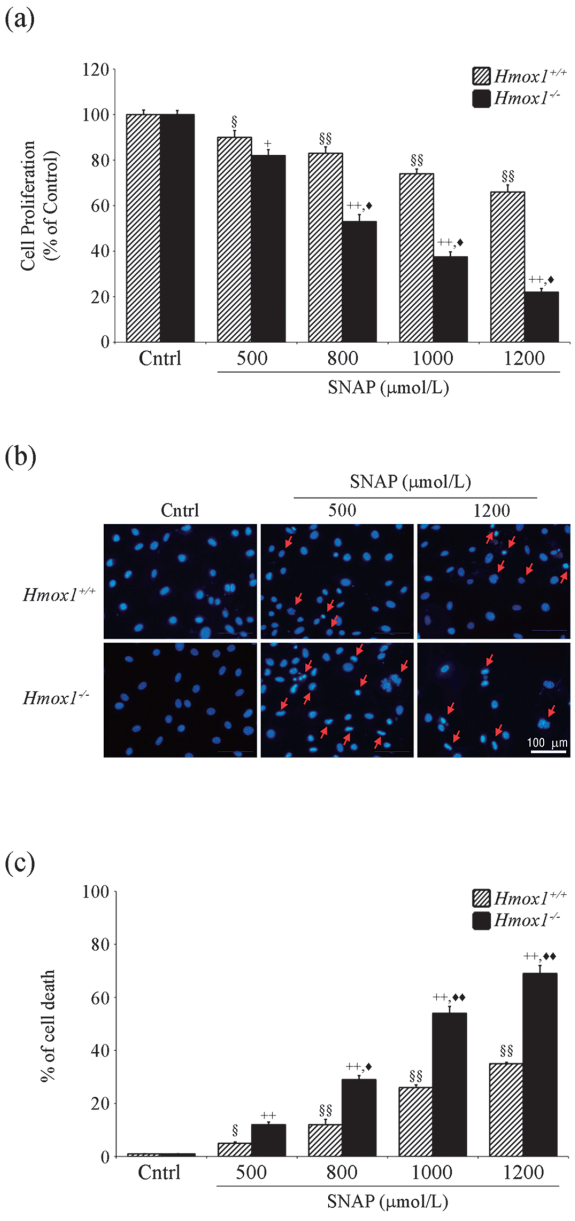

Fig. 5. HO-1 Prevents NO-Induced Cell Death in VSMC

Hmox1$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC treated with different concentrations of SNAP for 24 h. (a) Proliferation was assayed by BrdU incorporation and expressed as percentage of proliferating cells relative to control to which a value of 100% was arbitrarily assigned. Data represent the mean±S.D. of 4 independent experiments. (b) Representative photomicrographs of normal and apoptotic nuclei of cells stained by Hoechst 33258 dye evaluated under fluorescence microscopy at 20× magnification. Arrows indicate apoptotic figures. (c) Trypan blue exclusion test. Data are representative of 3 independent experiments and are expressed as mean±S.D. §,+ $p<0.05$ *vs.* Cntrl; §§,++ $p<0.01$ *vs.* Cntrl; ◆ $p<0.05$ *vs.* *Hmox1*$^{+/+}$; ◆◆ $p<0.01$ *vs.* *Hmox1*$^{+/+}$ VSMC.

ished HO-1 induction while allowed iNOS expression at 48 h ($p<0.01$ *vs.* Control). In contrast, *Hmox1*$^{-/-}$ VSMC treated with IL-1β and L-NAME (Fig. 4b) showed a marked increase in iNOS expression similar to treatment with IL-1β alone (Fig. 3b). Measurement of nitrite concentration in culture media confirmed that nitrite levels in both *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC were undetectable (Fig. 4c). These results confirm the hypothesis that NO induces HO-1 expression, which in turn regulates iNOS expression and NO production.

HO-1 Protects VSMC against NO-Induced Cell Death To determine whether NO-mediated growth inhibition can be regulated by HO-1, we treated *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC with different concentrations of the NO donor SNAP (Fig. 5a) to reach in the medium a range of NO concentrations similar to that supposed to be released from iNOS after balloon injury.[2] This resulted in a dose dependent decrease of cell proliferation in both *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC measured by BrdU incorporation ($p<0.05$, 500 μmol/l of SNAP *vs.* Control, and $p<0.01$, 800 through 1200 μmol/l of SNAP *vs.* Control). The effect was stronger in *Hmox1*$^{-/-}$ VSMC than in *Hmox1*$^{+/+}$ VSMC ($p<0.05$; Fig. 5a). Staining of cells with Hoechst dye incubated with SNAP revealed that the number of apoptotic cells increased in a dose dependent manner in *Hmox1*$^{-/-}$ VSMC ($p<0.05$, 800 μmol/l and $p<0.01$, 1000 to 1200 μmol/l of SNAP) more than in *Hmox1*$^{+/+}$ VSMC (Figs. 5b, c) supporting the hypothesis that the deficiency of HO-1 renders VSMC more susceptible to NO induced apoptosis. To further validate our proposed hypothesis, VSMC cells were incubated with different concentrations of SNAP for 24 h and apoptosis was detected using an anti-cleaved caspase-3 antibody (Fig. 6). Caspase-3 is activated in two steps generating an inactive fragment of 19 kDa (p19), which is further processed to the active 17 kDa (p17) fragment. Monitoring the caspase-3 p19/p17 ratio enables measurement of the activation of caspase-3.

Both *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC, cultured in normal medium (control) manifested presence of the 19 kDa fragment of caspase-3. Cells treated with H_2O_2, a pro-apoptotic stimulus, showed an increased level of apoptosis revealed by the increased presence of the active caspase-3 fragment (17-kDa) that marks the execution stage of apoptosis. Treatment of *Hmox1*$^{+/+}$ VSMC (Fig. 6a) with SNAP led to an enhanced apoptosis compared to control ($p<0.01$), measured as ratio of 17 kDa/19 kDa. Noteworthy, *Hmox1*$^{-/-}$ VSMC (Fig. 6b) showed a higher degree of apoptosis than the *Hmox1*$^{+/+}$ VSMC (Fig. 6a).

Since the level of the active form of caspase-3 protein expression is specifically regulated by X-linked inhibitor of apoptosis protein (XIAP), the role of this inhibitor in the control of cell apoptosis by HO-1 was examined. Our experiments showed that *Hmox1*$^{+/+}$ VSMC have increased cell death (+25% 1000 μmol/l and +34% 1200 μmol/l *vs.* Control $p<0.01$; Fig. 5c) accompanied by a decrease in XIAP protein after SNAP treatment ($p<0.05$ at 1000 μmol/l and $p<0.01$ at 1200 μmol/l *vs.* Control; Fig. 6a). Moreover, the down-regulation of XIAP was SNAP-dose-dependent in the *Hmox1*$^{+/+}$ cells. In *Hmox1*$^{-/-}$ VSMC, XIAP expression was found down regulated even in untreated cells (−29% compared to untreated *Hmox1*$^{+/+}$ VSMC; Figs. 6a, b) but probably sufficient to prevent cell death (Fig. 5c). When *Hmox1*$^{-/-}$ VSMC was treated with 500 μmol/l of SNAP, the expression of XIAP was lower (−21%) than the control as shown in Fig. 6b, resulting in increased apoptosis (+11% *vs.* Control $p<0.01$; Fig. 5c). Exposure of *Hmox1*$^{-/-}$ VSMC to higher concentrations of SNAP did not result in a further reduction of XIAP level but an increased rate of apoptosis was observed (+28% 800 μmol/l, +55% 1000 μmol/l and +68% 1200 μmol/l *vs.* Control $p<0.01$; Fig. 5c).

DISCUSSION

Neointimal formation is due to an exaggerated healing process that occurs in the vessel wall after a damaging lesion.

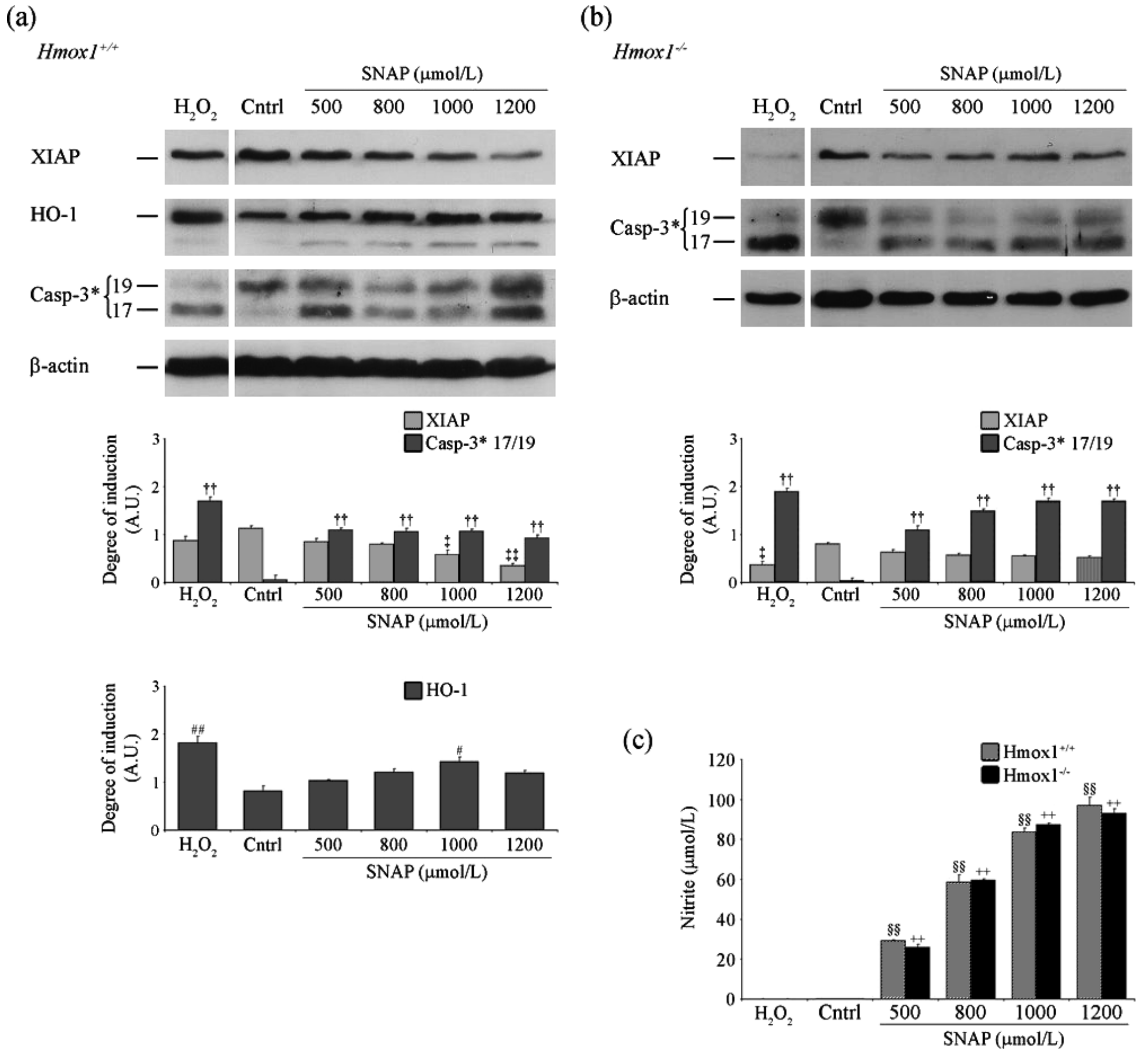

Fig. 6. Different Susceptibility of *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ VSMC to NO Toxicity

(a) *Hmox1*$^{+/+}$ and (b) *Hmox1*$^{-/-}$ VSMC were treated with different concentrations of SNAP for 24 h. In the control group cells were incubated with the medium alone; in H_2O_2 group cells were treated with H_2O_2 for 4 h. Cells lysates were harvested and XIAP, HO-1 and casp-3* were evaluated by Western blotting using specific antibodies. Quantification of the p17 and p19 fragments of caspase-3 was performed by densitometry. The values were normalized against the corresponding β-actin bands and then the ratio p17/p19 was calculated. β-Actin antibody was used to demonstrate equal protein loading. $^{\#,\ddagger}p<0.05$ *vs.* Cntrl; $^{\#\#,\ddagger\ddagger,\dagger\dagger}p<0.01$ *vs.* Cntrl. (c) The amount of NO released from SNAP in the culture medium was determined by Griess method. Data represent the mean±S.D. of 3 independent experiments. $^{++,\S\S}p<0.01$ *vs.* Cntrl. Casp-3*=cleaved caspase-3. A.U.=arbitrary units.

It is responsible for restenosis, limiting the success of many vascular interventions including balloon angioplasty with or without stenting.[1] Many studies suggest that oral L-arginine supplementation suppresses IH of the rat carotid artery after BI,[32,33] an effect that is potentiated by IL-1β.[31] The co-administration of L-arginine with L-NAME can reverse the L-arginine effect, indicating that NO, presumably through iNOS, mediates the attenuation of the IH.[33] HO-1, by exerting anti-inflammatory, antiproliferative, anti-apoptotic and anti-oxidant effects plays an important role in homeostasis of the vasculature.[34] Since NO has been shown to potently induce HO-1 expression in a variety of cell types[35,36] we further examined the role of HO-1 as a possible modulator of the pro-apoptotic effects of NO on VSMC.

We demonstrated, for the first time in a clinically relevant rat model of vascular injury, that treatment of animals with the natural NO precursor L-arginine prevents neointimal formation even when HO-1 activity is inhibited by SnPP-IX. These results further elucidate the role of proliferation/apoptosis of VSMC in the well-known HO-1-mediated vascular protection process, focusing on the NO contribution (Fig. 7). Others have recently shown, that SnPP-IX treatment, but without co-administration of L-arginine, completely restored neointimal formation abolishing the protective effect of HO-1.[19] Our working hypothesis from these findings is that NO has a role in preventing neointima formation that involves additional mechanisms other than those mediated by HO-1 inhibition of VSMC proliferation. The present study is consistent with a double role for L-arginine-NO in VSMC neointima formation.[37] First, NO inhibits VSMC proliferation. Indeed, in L-arginine treated rats, we observed a limited proliferation of VSMC with significant inhibition of intimal hyperplasia. Second, in the absence of HO-1, NO induces apoptosis of the proliferating VSMC. We demonstrated *in vivo* that NO decreases IH after BI in SnPP-IX-treated rats by increasing the rate of VSMC apoptosis. The lack of HO-1 activity led to a greater apoptotic cell death index along with concomitant higher cell proliferation recorded. This is in keep-

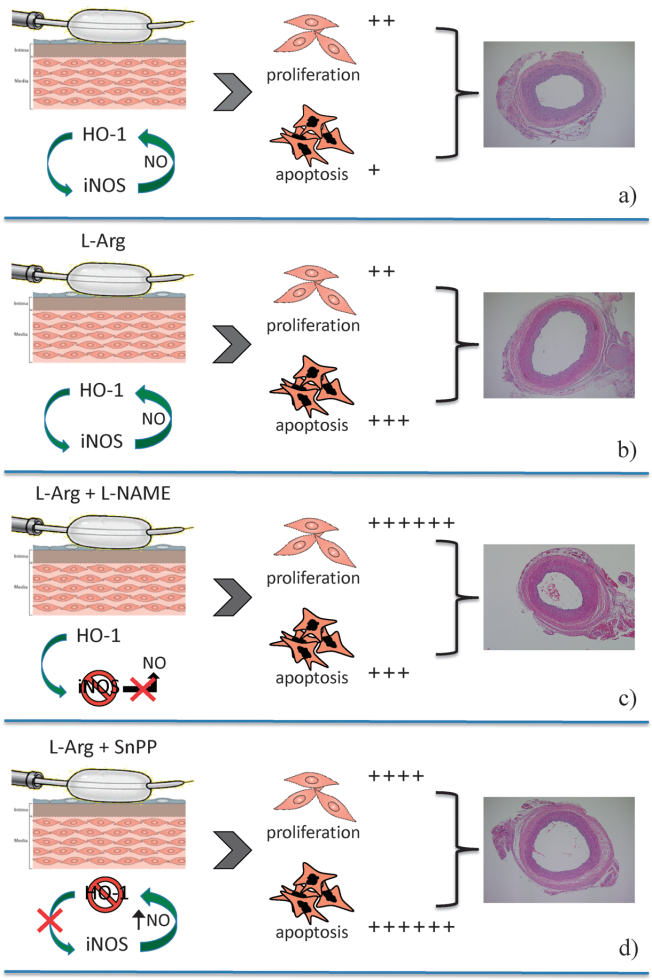

Fig. 7. Schematic Representation of Proposed Regulatory Interactions between NO and HO-1

(a) Balloon injury (group I) caused activation of both HO-1 and iNOS with increased cell proliferation and apoptotic cell death. The imbalance between cell growth and cell loss promoted intimal hyperplasia formation. (b) In balloon injured L-arginine treated rats (group II) the activation of HO-1 and iNOS was stronger than in group I, with sustained production of NO from L-arginine. iNOS-derived NO positively modulates HO-1. Activation of HO-1 leads to inhibition of cell proliferation. In the meantime, elevated levels of NO lead to an increased cell death preventing intimal hyperplasia formation. (c) Balloon injured rats treated with L-arginine together with L-NAME (group III); the NOS-derived NO was inhibited and proliferation was increased with enhanced neointima formation. (d) On the contrary, in balloon-injured rats treated with L-arginine together with SnPP-IX (group IV), the HO-1 activity was inhibited. Inactivation of HO-1 reduced its capacity to down-modulate iNOS-derived NO with the consequences of high levels of NO production and increased apoptosis rate. In the absence of HO-1, NO exerts therapeutic effects with a significant reduction in IH.

ing with a previous study showing the anti-proliferative effect of HO-1 in the artery *in vivo*.[9] In addition it has been demonstrated that apoptosis stimulates VSMC proliferation to replace those VSMC lost in response to injury.[38] However, our data showed that in the absence of HO-1, cell proliferation was unable to overcome less neointimal hyperplasia caused by apoptosis, achieving a significant reduction in the intimal area. These effects were probably due to the lack of the anti-apoptotic effect of HO-1. The present findings stress that NO can trigger apoptosis of VSMC in the vessel wall injured in rats in which HO-1 activity is inhibited, limiting the effects of the restenosis. On the other hand, the IH reduction is reversed with the administration of L-NAME hampering the L-arginine effects.

To better clarify the effect of HO-1 on the expression of iNOS, $Hmox1^{+/+}$ and $Hmox1^{-/-}$ VSMC were treated with IL-1β to induce endogenous production of NO. In $Hmox1^{+/+}$ VSMC the results suggested that HO-1 could modulate IL-1β induced iNOS expression and activity.[39] In $Hmox1^{-/-}$ VSMC, the iNOS expression and NO production were enhanced and sustained, supporting the hypothesis that in the absence of HO-1 the activity of iNOS is no longer requested. This is also consistent with *in vivo* results concerning the concentration of nitrite in plasma of SnPP-IX treated animals.

Because NO has been shown to modulate HO-1 expression[29,40,41] its role was investigated in $Hmox1^{+/+}$ and $Hmox1^{-/-}$ VSMC co-treated with IL-1β and with an inhibitor of NO production, L-NAME. The NO inhibitor abrogated the ability of iNOS to produce NO, preventing HO-1 induction in $Hmox1^{+/+}$ VSMC.

Since there is a delicate balance in the vessel wall between apoptosis and proliferation, any perturbation may contribute to vascular remodeling. NO is one of the major factors responsible for vascular homeostasis: it maintains the vascular tone and regulates smooth muscle cell phenotype and proliferation; any deviation from physiological concentrations of NO results in a modification of the VSMC proliferation state. Impaired vascular wall integrity during endothelial denudation induces an inflammatory response associated with an increase of vascular superoxides. Superoxides, sequestering NO, drastically reduce its bioactivity and its bioavailability.[42] These phenomena cause VSMC activation and migration from the tunica media to the intima, where their proliferation and increased synthetic potential lead to the formation of the neointima lesion.[43] Delivery of exogenous NO reduces the sequelae of acute inflammation and suppressed intimal hyperplasia formation as shown previously.[44] In this context NO seems able to induce VSMC apoptosis, which has an important role in the prevention of neointimal formation.[45—48] Based on the findings that NO induces apoptosis we analyzed whether a NO donor would exert similar effects on $Hmox1^{+/+}$ and $Hmox1^{-/-}$ VSMC. We found that SNAP caused a dose dependent decrease of cell proliferation even though this effect was more evident in $Hmox1^{-/-}$ than in $Hmox1^{+/+}$ VSMC ($p<0.05$). The changes in nuclear morphology showed by Hoechts staining revealed an increase in the number of apoptotic cells, more visible in $Hmox1^{-/-}$ than in $Hmox1^{+/+}$ VSMC.

It is widely assumed that activation of apoptotic nucleases together with caspase-mediated cleavage of a number of downstream nuclear proteins is responsible for the characteristic changes in nuclear morphology that include chromatin condensation and nuclear fragmentation. Thus, we investigated whether caspase-3 was activated in both $Hmox1^{-/-}$ and $Hmox1^{+/+}$ cells treated with different concentrations of SNAP. These treatments led to caspase-3 cleavage in both $Hmox1^{+/+}$ VSMC and $Hmox1^{-/-}$ VSMC. Interestingly, the 17 kDa fragment, the executioner marker of apoptosis, appears to prevail in $Hmox1^{-/-}$ VSMC *vs.* $Hmox1^{+/+}$ VSMC. This result pointed out that the proteolytic process of caspase-3 in $Hmox1^{-/-}$ VSMC in response to NO can be primed and apoptosis completed compared to $Hmox1^{+/+}$ VSMC suggesting an additional role for HO-1 in regulation of apoptosis. Caspase activation and its prevention are subject to very tight control by many cellular factors. Members of the IAP

(inhibitor of apoptosis) protein family have been shown to bind directly to specific caspases and suppress apoptosis. XIAP is the most potent IAP protein and is known to suppress cell death by binding to and inhibiting caspase-3.[49] In this regard, a decrease of XIAP levels released the inhibition of apoptotic process, at least in part through activation of procaspases, and the increase of XIAP may be an important element in determining the fate of the cell.[50] Our experiments showed that in *Hmox1*$^{+/+}$ VSMC the increase in apoptosis was accompanied by a decrease in XIAP protein after SNAP treatment. The down-regulation of XIAP was SNAP-dose-dependent in the *Hmox1*$^{+/+}$ cells. In contrast, the *Hmox1*$^{-/-}$ VSMC showed low expression of XIAP when treated with different concentrations of SNAP compared with control. This tight control over cellular XIAP activity demonstrates the importance of this protein in maintaining a proper apoptotic threshold within the cell. The HO-1 deletion in VSMC increased the endogenous stress pressure sustained from a decrease of XIAP expression making the cells more susceptible to caspase-3 mediated apoptosis.

Restenosis remains the most important long-term limitation to angioplasty and stenting in cardiovascular disease. Different studies support the therapeutic use of L-arginine derived NO in the prevention of restenosis[32,33] but there are obstacles to be addressed before its full translation into clinical practice.[51] Our results provide new insight into mechanisms by which NO induces a protective mechanism in the prevention of IH suggesting that NO may exert two different effects on VSMC, pro-apoptotic *versus* cell cycle/regulation and these effects seem to be mediated by HO-1 (Fig. 7).

Acknowledgements This work was supported by Grants from MIUR (PRIN 2004, N. 2004060070 to M.L.), and F.A.R. 2004, 2005, 2006—University of Milano-Bicocca (to M.L.).

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