Expression and distribution of heme oxygenase-1 and -2 in rat brown adipose tissue: the modulatory role of the noradrenergic system

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Abstract To investigate whether brown adipose tissue (BAT) expresses the inducible (HO-1) and the constitutive (HO-2) isoform of heme oxygenase, reverse transcriptase-polymerase chain reaction, Western blotting and immunohistochemistry were performed on interscapular BAT (IBAT) from rats acclimated at environmental temperature or exposed to cold. Both HO isoforms were detected in rat IBAT. They were immunolocalized in the cytoplasm and/or nuclei of brown adipocytes, in parenchymal capillaries, arteries and in some veins and nerves. Whereas cold exposure did not affect HO-2 expression, it significantly increased the expression of HO-1, both at mRNA (about 3-fold) and protein (about 2-fold) levels, reflecting the increased expression of HO-1 in the brown adipocytes and endothelial cells of parenchymal capillaries. Western blotting of cytosolic and nuclear protein extracts from cultured differentiated brown adipocytes showed that HO-1 and HO-2 are indeed localized in the cytosol and nuclei of brown adipocytes, and that noradrenaline stimulation significantly increased their amount in cytosol but not in the nuclear fraction.

Key words: Heme oxygenase; Heat shock protein 32; Brown adipose tissue; Noradrenaline; Rat

1. Introduction

As a major site of both non-shivering and diet-induced thermogenesis, the mammalian brown adipose tissue (BAT) plays an important role in regulating the energy balance [1–3]. Its rich sympathetic innervation and vascularity are related to functional and pathological conditions. In particular, noradrenergic function and vasodilation are stimulated by cold exposure [1] and inhibited in thermoneutral conditions (28°C for rats) as well as in some syndromes of rodent genetic obesity [4] where reduction in energy expenditure is thought to be involved in pathogenesis. It has recently been shown that brown adipocytes express different isoforms of nitric oxide synthase (NOS) and thus synthesize and release nitric oxide (NO) via noradrenergic stimulation [5,6]. NO seems to be involved in the sympathetic induction of BAT vasodilation [5] to match thermogenesis with perfusion, as well as in the proliferation and differentiation of brown adipocytes in vitro [7].

Recent evidence suggests the existence of a close relationship between the anatomical distribution and the biological functions of NOS and heme oxygenase (HO) [8]. HO is a ubiquitous microsomal enzyme which produces a newly identified gaseous mediator, carbon monoxide (CO), and plays a crucial role in maintaining cellular heme homeostasis and hemoprotein levels [9,10]. HO consists of two homologous isozymes, the constitutively expressed HO-2 and the inducible HO-1. The latter, also known as heat shock protein (HSP) 32, is induced by a wide range of stimuli that cause oxidative stress and pathological conditions such as heat shock, hypoxia, hyperoxia and cellular transformation [11,12]. Based on these premises, we set out to study whether the HO system might be expressed in BAT and have a role in brown fat function. To do this, we investigated the expression of the two HO isoforms in the interscapular BAT (IBAT) of rats acclimated at 22°C or exposed to 4°C for 2 days: by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis to evaluate in vivo their mRNA and protein levels, and by immunohistochemistry to detect their cellular localization. The expression of HO-1 and -2 was also investigated in rat cultured brown adipocytes. Results showed that: (1) both isozymes are clearly detectable in rat IBAT, mainly in the cytoplasm and/or nuclei of brown adipocytes, in parenchymal capillaries and extra- and intralobular arteries; (2) capillary and brown adipocyte HO-1, but not HO-2, levels are upregulated following cold exposure; and (3) HO-1 and -2 are detected in the cytosolic and nuclear protein extracts of cultured differentiated brown adipocytes, where noradrenaline stimulation upregulates their levels in cytosol, but not in the nucleus. On the whole, these findings suggest that the HO system may be involved in brown fat function.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats 6 weeks of age obtained from Morini Laboratories (S. Polo d'Enza, Italy) were individually caged and kept at 22°C or exposed to 4°C for 2 days. All animals had free access to food and water. Lights were on for 12 h daily (07.00–19.00). Animal care was in accordance with institutional guidelines and the experimental protocol was approved by the local Animal Care Ethical Committee.

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2.2. RT-PCR analysis

Total RNA was isolated from 20 mg of IBAT using the RNazol method (TM Cinna Scientific, Friendswood, TX, USA). RT-PCR was performed as previously described [13]. A control experiment without reverse transcriptase was performed on each sample in order to exclude that amplification was due to residual genomic DNA. An aliquot (10% vol) of the cDNA was amplified using the specific primers for rat HO-1, HO-2 and GAPDH [14] by *HotStarTaq* DNA polymerase (Qiagen, Genenco, Florence, Italy) in 25 μl of standard buffer, 2.5 mM $MgCl_2$ and 200 μM dNTPs. *HotStarTaq* DNA polymerase requires an activation step of 15 min at 95°C. The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 58°C for 40 s and polymerization at 72°C for 40 s. After 30 cycles a final 10 min incubation at 72°C was carried out. Ten μl of the PCR products was separated by electrophoresis (2.0% agarose gel in Tris–acetate–EDTA buffer, containing 0.1 mg/ml of ethidium bromide), revealed with a QuickImage-D (Canberra Packard, Milan, Italy) and densitometrically analyzed with Phoretix 1D, version 3.0 (Phoretix International, UK) [13].

2.3. Cell culture and treatment

Brown fat precursor cells were isolated from IBAT as described previously [15,16]. Three million cells seeded in 6-well culture plates (Nunclon Delta, Milano, Italy) were cultured in a water-saturated atmosphere of 6% CO_2 in air at 37°C in 2.0 ml of a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 10% newborn calf serum, 4 nM insulin, 4 nM triiodothyronine, 10 mM HEPES, 50 IU penicillin, 50 μg streptomycin, and 25 μg sodium ascorbate per ml (all from Flow Laboratories, Milan, Italy). On day 8 (i.e. at the time of confluence and differentiation), 10 μM noradrenaline was added to the culture for 8 h for Western blot analysis.

2.4. Cell fractionation

The procedure used was based on a method described by Bulbarelli et al. [17]. Briefly, cells grown on Petri dishes were washed free of medium with cold phosphate-buffered saline containing 5 mmol/l EDTA, incubated in the same buffer for 5 min on ice, detached with a rubber policeman, and collected by centrifugation. The pellet was resuspended in cold hypotonic buffer (1 mmol/l EDTA, 15 mmol/l KCl, 30 mmol/l NaCl, 1 mmol/l Tris–HCl, pH 7.5) containing 1 mmol/l PMSF plus the Sigma (Milan, Italy) cocktail of protease inhibitors. After 5 min, cells were ruptured by passage through a sterile syringe (1 ml, 26GA 3/8). The volume of the homogenate was measured and the same volume of cold, two times concentrated, isotonic buffer (0.5 mol/l sucrose, 0.2 mmol/l EDTA, 2 mmol/l Tris–HCl, pH 7.4) was added. The solution was centrifuged at 500×*g* for 10 min at 4°C to obtain a nuclear pellet and a postnuclear supernatant (PNS). Monoclonal anti-β-tubulin (Sigma) recognizes a specific protein of 55 kDa in PNS, but not in nuclear preparations (data not shown).

2.5. Antibodies

Western blotting and immunohistochemistry were performed with two sets of antibodies: (1) rabbit polyclonal antibodies against HO-1 and -2 (StressGen Biotechnologies Corp., Victoria, Canada); (2) rabbit polyclonal antibody against HO-1 (ABR, Golden, CO, USA) and goat polyclonal antibody against HO-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All antibodies were specifically recommended by the manufacturer for both Western blotting and immunohistochemical analysis. No differences were detected between the antibodies under (1) and (2) in Western blotting, whereas in immunohistochemistry the former antibodies yielded a better specific staining and less background.

2.6. Western blotting

Western blotting was performed as described [18]. IBAT was obtained from five rats of each group killed by decapitation. For HO-1 and -2 analysis, 50 μg and 15 μg of proteins, respectively, were boiled and resolved by 12% SDS–PAGE under reducing conditions and transferred to nitrocellulose filter paper. Incubation with the primary antibodies (see Section 2.4) was performed for 1 h at room temperature in TBS-T with 3% milk at a dilution of 1:1000. After multiple washes in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated IgG secondary antibody (Pierce, Milano, Italy) at 1:100 000 dilution and the signal was detected on Biomax film (Kodak, Milano, Italy) with the enhanced chemiluminescence kit (Pierce). For quantitation, densitometric measurements were performed with Phoretix 1D, version 3.0. Statistical significance was determined by ANOVA test.

2.7. Immunohistochemistry and morphometry

Five rats from each group were euthanized under intraperitoneal anesthesia (100 mg/kg ketamine-Ketavet, Farm. Gellini, Aprilia, Italy, in combination with 19 mg/kg xylazine-Rompum, Bayer AG, Leverkusen, Germany) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. IBAT was dissected and postfixed overnight in the same fixative by immersion. Samples were then dehydrated and paraffin-embedded. Immunoreactivity was assessed in 3 μm thick serial IBAT sections by the avidin-biotin-peroxidase method as previously described [19]. Negative controls were obtained in each instance by omitting the primary antibody and using preimmune serum instead of the primary antiserum. To test antibody specificity, immunohistochemical reactions were performed in parallel on tissues known to contain the antigens: spleen for HO-1 [20] and

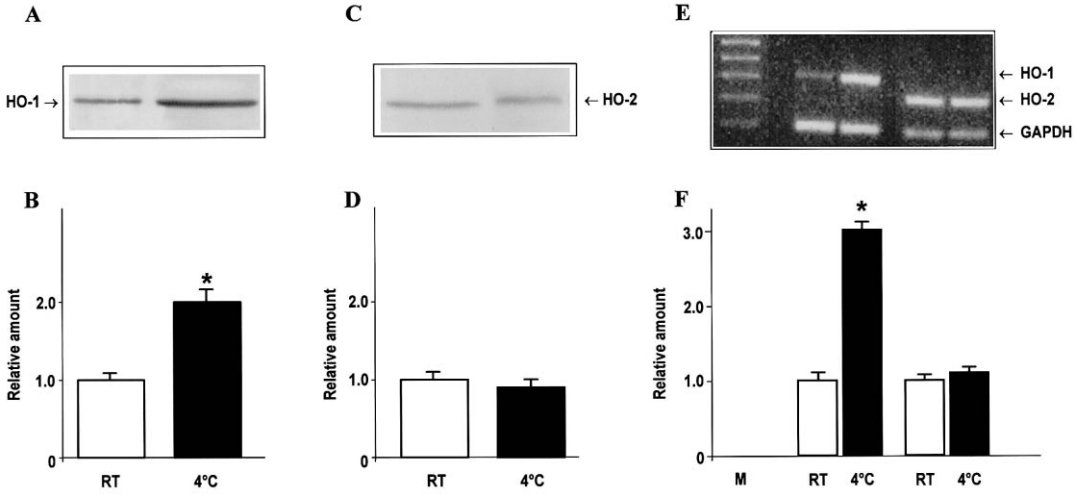

Fig. 1. Western blot (A and C) and RT-PCR (E) analyses of heme oxygenase-1 (HO-1) and -2 (HO-2) in brown fat from rats kept at 22°C (room temperature, RT) or 4°C for 2 days. A and C: Immunoblot obtained by separating 50 (HO-1) and 15 (HO-2) μg on 12% SDS–polyacrylamide gel under reducing conditions. E: Ethidium bromide agarose gel electrophoresis of HO-1 and HO-2 cDNA fragments amplified by RT-PCR. M, molecular weight marker. B, D and F: Densitometric analysis of three immunoblotting and RT-PCR experiments. Bars represent means ± S.E.M., plotted relative to HO-1 or HO-2 in brown fat of animals kept at RT taken as 1. *$P < 0.05$ vs. RT.

testis for HO-2 [21]. For each antibody, several different dilutions were tested in order to obtain the maximum specific reaction product with the lowest background. Morphometric analysis of HO-positive nuclei was performed on three representative sections from three animals of each experimental group. For each section, 10 high-power fields were randomly selected and the percentage of positive nuclei vs. the total number of brown adipocyte nuclei was calculated. Mean values ± S.E.M. are given in the text. Statistical significance was determined using Student's paired *t*-test.

3. Results

3.1. Heme oxygenase-1 (inducible isoform)

3.1.1. Rats kept at 22°C. Expression of HO-1 mRNA was revealed by RT-PCR (Fig. 1E). Furthermore, rat IBAT contained a protein species of approximately 32 kDa, which cross-reacted with polyclonal anti-HO-1 antibodies (Fig. 1A). Immunohistochemistry showed that HO-1 was expressed in some extralobular nerves, in smooth muscle cells and some endothelial cells of extra- and intralobular arteries, in some parenchymal capillaries and brown adipocytes. In the latter, the reaction product was usually detected in the cytoplasm, but some adipocytes also exhibited nuclear staining (Fig. 2, upper left).

3.1.2. Rats exposed to 4°C. IBAT HO-1 mRNA levels were significantly higher (about 3-fold) in cold exposed animals then in rats acclimated at environmental temperature (Fig. 1E and F). Densitometric analysis of immunoblotted samples confirmed that HO-1 protein content increased by 100 ± 8% after cold exposure (Fig. 1B). Accordingly, immunohistochemistry showed that capillaries as well as brown adipocytes stained more intensely and diffusely (Fig. 2, upper right). Brown adipocyte staining was stronger in the cytoplasm and the number of positive nuclei was more than double that observed in rats kept at 22°C (22 ± 3% vs. 9 ± 1%, $P < 0.05$).

3.1.3. Cultured brown adipocytes. Brown fat precursors grew in culture and divided rapidly. At confluence (8–10 days), they appeared differentiated and resembled mature brown adipocytes. Western blot analysis performed on these cells on day 8 showed HO-1 in both cytosolic and nuclear

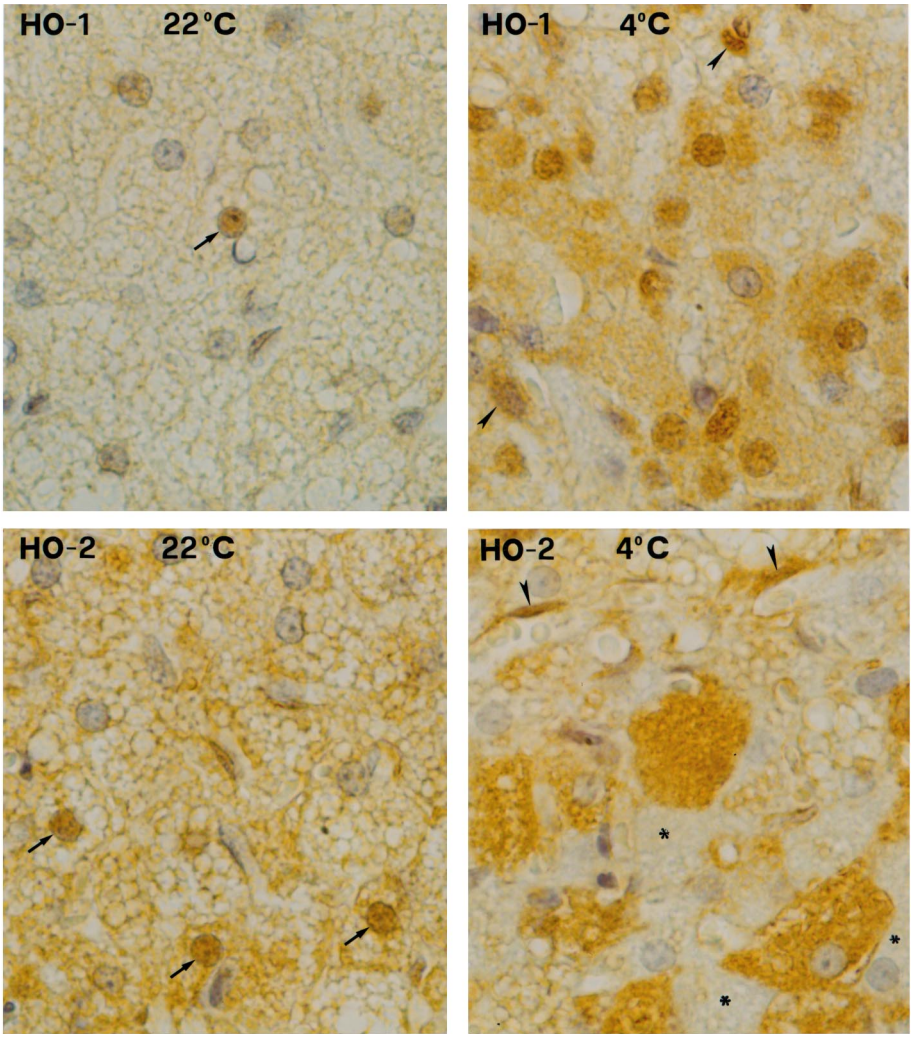

Fig. 2. Immunohistochemical analysis of heme oxygenase-1 (HO-1) and -2 (HO-2) in rat brown fat (rabbit polyclonal antibodies from Stress-Gen Biotechnologies). In rats kept at 22°C, brown adipocytes were weakly positive for HO-1; some brown adipocytes also exhibited weak nuclear staining (arrow). After cold exposure (4°C for 2 days), capillaries (arrowheads) and brown adipocyte cytoplasm and nuclei stained more diffusely and intensely. In rats kept at 22°C, brown adipocytes stained for HO-2 in the cytoplasm and some nuclei (arrows); capillaries were also positive. After cold exposure, intensely HO-2-positive brown adipocytes were scattered among weakly stained or negative (asterisks) adipocytes. Capillaries were positive (arrowheads). 1075×.

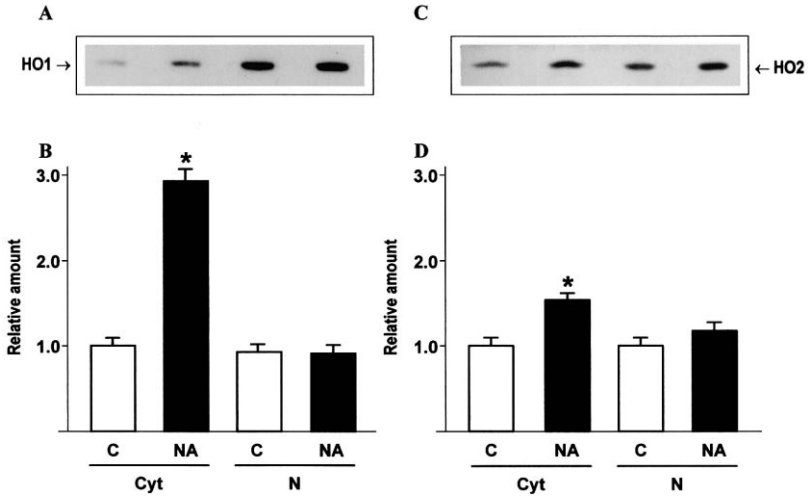

Fig. 3. Western blot analysis of heme oxygenase-1 (HO-1) (A) and -2 (HO-2) (C) in cytosolic (Cyt) or nuclear (N) protein preparations of rat differentiated brown adipocytes on day 8 of culture exposed to saline (C) or 10 μM noradrenaline (NA) for 8 h. Bars represent means ± S.E.M., plotted relative to HO-1 or HO-2 in cytosolic or nuclear protein of saline-treated brown fat cells taken as 1. $*P < 0.05$ vs. saline.

protein extracts (Fig. 3A). After treatment with 10 μM noradrenaline for 8 h, HO-1 significantly increased in cytosol extracts, but remained unchanged in the nuclear extract (Fig. 3B).

3.2. Heme oxygenase-2 (constitutive isoform)

3.2.1. Rats kept at 22°C. The expression of HO-2 mRNA was revealed by RT-PCR (Fig. 1E). Protein analysis showed that rat IBAT also contained a protein species of approximately 36 kDa, which cross-reacted with polyclonal anti-HO-2 antibodies (Fig. 1C). In rats kept at 22°C, extra- and intralobular arteries, some extralobular nerves and veins, brown adipocytes and capillaries stained for HO-2. As observed for HO-1, in positive brown adipocytes the reaction product was usually detected in the cytoplasm; in some the nucleus was also positive and in a few of them only the nucleus was positive (Fig. 2, lower left).

3.2.2. Rats exposed to 4°C. No significant differences in IBAT HO-2 mRNA (Fig. 1E and F) and protein (Fig. 1D) levels were observed between rats exposed to cold and those kept at 22°C. Immunohistochemistry revealed that after cold exposure intensely positive brown adipocytes were scattered among negative or weakly positive adipocytes (Fig. 2, lower right). Capillaries were positive and the number of positive brown adipocyte nuclei was not significantly different from that observed in rats kept at 22°C (25 ± 3% vs. 27 ± 5%, $P > 0.05$).

3.2.3. Cultured brown adipocytes. Western blotting of differentiated brown adipocytes showed HO-2 in both cytosolic and nuclear protein extracts (Fig. 3C). After treatment with 10 μM noradrenaline for 8 h, HO-2 significantly increased in the cytosol, but remained unchanged in cell nuclei (Fig. 3D).

4. Discussion

In the present study we show for the first time that rat brown fat contains substantial amounts of both the inducible and the constitutive isoform of HO. Specific immunostaining was mainly found in brown adipocytes, parenchymal capillaries and blood vessels. BAT thermogenesis is overwhelmingly regulated by the sympathetic nervous system [1–3]. The increase in HO-1 expression detected after cold exposure suggests that the expression of this enzyme isoform is regulated by brown fat noradrenergic tone, similarly to NOS [5,6] and to other molecules involved in the physiological activation of brown fat. This result was confirmed by the significant increase in cytosolic HO-1 expression observed in differentiated brown adipocytes in culture following treatment with noradrenaline. Morphometric analysis showed that, in vivo, cold exposure also induced a significant increase in the number of HO-1-positive nuclei. By contrast, noradrenaline treatment did not affect HO-1 levels in the nuclei of cultured brown adipocytes. One possible explanation for this is that, in vivo, permissive factors might regulate the translocation of HO from cytosol to cell nuclei. The increased HO-2 expression observed in the cytosol of noradrenaline-treated brown adipocytes seems to contrast with the in vivo data showing non-significant differences between rats kept at 22°C and cold-exposed animals. This finding is however in line with the increased cytoplasmic immunostaining observed in vivo in some brown adipocytes, which were probably preferentially activated by the cold stimulus.

The stress-inducible protein HO-1 (HSP32) and the constitutive isozyme HO-2, which are different gene products, catalyze the oxidation of heme to molecules that are currently being implicated in important biological functions: iron, biliverdin and CO. Iron released by HO activity regulates genes, including those of NOS [22]; bilirubin, which is formed when biliverdin is reduced by biliverdin reductase, is a potent antioxidant [23]; finally, CO is thought to be, like NO, a gaseous signal molecule for generation of cGMP in biological systems [24]. Like NO, CO has a high diffusible capacity but a short half-life [10,25,26] and is hypothesized to act at the sites where it is produced. It follows that immunohistochemical detection of the localization of the HO isoforms allows to surmise their putative targets, and to speculate about their possible functions in cells and tissues.

CO and NO induce vasodilation in several vascular beds [27,28]. In this context, the presence of HO in IBAT smooth muscle cells of the arteries, in the endothelial cells of parenchymal capillaries and in brown adipocytes appears to be strategic for the regulation of vascular tone and blood flow

through this tissue. The increased expression of the inducible isoform observed in cold-exposed rats well correlates with the increase in blood flow required to tackle the changed environmental conditions [29].

In serial sections from animals acclimated to cold temperature for different periods of time, HO-1 is usually, or more intensely, expressed, in the same brown adipocytes that are also positive, or more intensely positive, for UCP1 (our unpublished data), the thermogenic mitochondrial uncoupling protein [1,3,30]. Noradrenaline-induced mitochondrial uncoupling and heat production in brown fat cells exceed by several times the heat produced in any other cell type. Thus, brown adipocytes are subject to considerable risk of oxidative and heat-induced damage. We speculate that the increased expression of HO-1 observed after cold exposure in the thermogenically more active brown adipocytes could protect these cells, both directly through maintenance of cellular heme homeostasis and hemoprotein levels – heme molecule is the most effective promoter of lipid peroxidation and oxygen free radical formation, which exert adverse effects on cells [11,12,23] – and indirectly through vasodilation (a common tissue protection mechanism).

Finally, the nuclear staining for both HO isoforms, confirmed by their detection in nuclear protein preparations by Western blotting, and immunohistochemical changes reflecting the different degree of brown fat functional activation (more numerous HO-1-positive nuclei in cold-exposed rats) point to a putative role for HO byproducts, specifically CO, as transcription factors, able to modulate the expression of genes involved in adipogenesis and thermogenesis, as already suggested for NO [7].

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