Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus

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Abstract The intracellular localization and activity of the nitric oxide synthase (NOS) isoforms were investigated in rat brown adipocytes. Immunohistochemistry showed cytoplasmic and nuclear staining for the endothelial NOS (eNOS) and inducible NOS (iNOS) isoforms; accordingly, anti-L-citrulline antibody, a marker of NOS activity, immunostained both the cytoplasm and the nucleus. The presence of metabolically active NOS in the nucleus was further confirmed by immunoblotting analyses of subcellular fractions of homogenates from cultured brown adipocytes and by measurements of NOS activity in the cytosol and nucleus. Sympathetic stimulation in vivo (i.e. cold exposure or β_3-adrenergic agonist treatment) and in vitro (i.e. noradrenaline treatment of cultured cells) significantly increased both cytosolic and nuclear eNOS and iNOS expression and activities. By contrast, the number of iNOS-positive, but not eNOS-positive, nuclei was significantly lower in the functionally impaired brown fat of genetically obese Zucker fa/fa rats. These data suggest the existence of a noradrenaline-modulated functional NOS system in the nucleus of brown adipocytes.

Key words: Nitric oxide synthase; Brown adipocyte; Cell nucleus; β_3-adrenoceptor; L-citrulline

1. Introduction

In mammals, brown adipose tissue (BAT) is a strategically located tissue whose primary function is to disperse the energy contained in lipids as heat (non-shivering thermogenesis) [1,2]. The thermogenic activity of brown adipocytes is due to the presence in mitochondria of a specific protonophoric protein, uncoupling protein 1 (UCP1) [3], and is triggered by noradrenaline (NA), which is released from the sympathetic nerves and acts mainly via β_3-adrenoceptors in brown adipocytes [1,2,4].

Nitric oxide (NO) is a gaseous messenger molecule that has paracrine and autocrine functions [5–7]. Three NO synthase (NOS) isozymes have been well characterized so far: the Ca^{2+}/calmodulin-dependent endothelial NOS (eNOS) and neuronal NOS (nNOS) isoforms and the Ca^{2+}/calmodulin-independent inducible NOS (iNOS) isoform [8]. In BAT, NO is involved in the vasodilation that matches heat production with tissue perfusion [9,10]. Of note, NA stimulates NO production and release from cultured brown adipocytes, and iNOS can be dynamically modulated in BAT in vivo since acute cold exposure augments through increased NA output [1,2,4] both iNOS mRNA and NOS activity [10].

Given that NO is a short-lived molecule that can diffuse freely across biological structures but acts within a restricted volume [11,12], the cellular and tissue localization of NOS isoforms can provide crucial insights into the physiological role of NO. We thus undertook an immunohistochemical study of the distribution of the NOS isoforms in BAT. To our surprise, we found eNOS- and iNOS-specific staining in the nuclei of brown adipocytes. This prompted us to design a study to assess whether functional NOS systems could exist in the nucleus of these cells and be modulated under conditions of different functional activation.

2. Materials and methods

2.1. Animals and treatments

Six-week-old male Sprague–Dawley rats (Charles River, Calco, Italy) were fed and housed in standard conditions (22°C). Some received the NOS inhibitors *N*-iminoethyl-L-lysine (L-NIL) and *N*-methyl-L-arginine (L-NMMA; both from Sigma, Milan, Italy) for 2 days (respectively, 3 and 30 mg/kg/day, i.p.) before immunohistochemical evaluation. Some individually caged rats were acclimated to 4°C for 3 or 14 days. Another group received 1 mg/kg/day of CL 316,243, a selective β_3-adrenoceptor agonist (kindly provided by K. Steiner, Wyeth-Ayerst, USA), i.p. for 7 days. Finally, 6-week-old genetically obese male Zucker rats (fa/fa) and their lean littermates (+/fa) were obtained from Harlan Nossan (Corezzana, Italy).

2.2. Antibodies

Immunohistochemistry and Western blotting were performed using two antibody sets: rabbit polyclonal antibodies against iNOS, eNOS and nNOS (BioMol Research Laboratories, Plymouth Meeting, PA, USA) and against L-citrulline (Arnel, New York, NY, USA), and monoclonal antibodies against iNOS, eNOS and nNOS (Transduction Laboratories, Lexington, KY, USA). We confirmed antibody specificity in Western blot preparations from positive controls and BAT. There were no differences between the antibodies at Western blotting; at immunohistochemistry, the polyclonal antibodies yielded better specific staining and less background.

2.3. Immunohistochemistry and morphometry

Rats were killed under i.p. anesthesia (100 mg/kg ketamine (Ketavet), Farm. Gellini, Aprilia, Italy, in combination with 19 mg/kg xylazine (Rompun), Bayer AG, Leverkusen, Germany) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Interscapular BAT was dissected and postfixed overnight in the same fixative by immersion. The samples were then dehy-

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drated and paraffin-embedded. Immunoreactivity was assessed in 3-μm-thick serial sections using the avidin–biotin–peroxidase method as already described [13]. To better appreciate negative nuclei, sections were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibody, using preimmune serum instead of the primary antiserum and adsorbing the antibody with an excess of antigen. Positive controls were: lipopolysaccharide (LPS)-treated liver for iNOS, cerebellum for eNOS, and skeletal muscle for nNOS. The antiserum against L-citrulline was raised against a keyhole limpet hemocyanin conjugate of L-citrulline and its specificity was evaluated in rat cerebellum [14]. The number of iNOS- and eNOS-positive nuclei was morphometrically analyzed on five representative sections from three rats per experimental group. In each section, we evaluated the percentage of positive nuclei vs. the total number of brown adipocyte nuclei in ten randomly selected high-power fields.

2.4. Brown adipocyte culture and drug treatments

Brown fat precursor cells and intact brown adipocytes were isolated as described previously [15] with some modifications [10]. Cells were exposed for 24 h to NA (1 μM, freshly diluted in buffers containing 0.1% ascorbic acid) or tumor necrosis factor-α (TNF-α; 10 nM) and then harvested.

2.5. Cell fractionation and assay of NOS activities

The procedure for cell fractionation was as described previously [16]. Cross-contamination of the nuclear fractions with other organelles or cytosolic enzymes was evaluated by immunodetection of the following markers: β-tubulin (monoclonal antibody, 1:500; Sigma), Na,K-ATPase α_1 subunit (monoclonal antibody, 1:500; kindly provided by Dr. G. Pietrini, Milan University, Italy) [17], ribophorin I (Rib I, polyclonal antibody, 1:150; kindly provided by Prof. N. Borgese, Catanzaro University, Italy) [18], cytochrome *c* oxidase subunit IV (COX IV, monoclonal antibody, 1:5000; Molecular Probes, Eugene, OR, USA), histone H1 (H1, monoclonal antibody, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Brown adipocyte eNOS and iNOS activities were estimated by measuring the conversion of L-2,3,4,5-[^{3}H]arginine to L-2,3-[^{3}H]citrulline. Ten million brown adipocytes, untreated or treated with 1 μM NA for 24 h, were homogenized using an Ultra-Turrax homogenizer (5 mm blade) for 20 s in 0.5 ml of a buffer containing 50 mM HEPES, pH 7.4, 1 mM DL-dithiothreitol, 1 mM EDTA (for iNOS), 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (12 000 × *g* for 20 min at 4°C), an aliquot of the supernatant was added to the reaction mixture to reach a final volume of 100 μl containing 50 mM HEPES, pH 7.4, 50 nCi L-[^{3}H]arginine (310 mCi/mmol; Amersham, Milan, Italy), 1 μM arginine, 1 mM NADPH, 10 μM $CaCl_2$ (for eNOS), 1 mM EDTA and 1 mM EGTA (for iNOS), 10 μM FAD, 0.1 mM (6*R*)-5,6,7,8-tetrahydro-1-biopterin. The mixture was incubated at 37°C for various periods of time. The reaction was stopped by adding 0.4 ml of a 1:1 slurry of Dowex AG 50W-X8 (Na^+ form, Fluka, Milan, Italy) in 50 mM HEPES, pH 5.5; supernatant radioactivity was measured after 15 min of shaking. Enzyme activities were expressed as nmol of citrulline formed by 1 mg of protein.

2.6. Western blotting

For eNOS and iNOS analyses, 50 and 100 μg, respectively, of cell fraction proteins were resolved through 8% SDS–polyacrylamide gel electrophoresis under reducing conditions, and Western blotting was performed as described previously [10]. Samples were densitometrically quantitated with a Quick Image densitometer (Canberra, Packard, Milan, Italy) and a Phoretix 1D, version 3.0, software image analyzer.

2.7. Data analysis

Data are reported as mean values ± S.E.M.; all the experiments were performed at least three times, each time with three or more independent observations. Comparisons were made using one-way analysis of variance followed by Student–Newman–Keuls post hoc comparisons; *P* values of < 0.05 vs. the controls were considered significant.

3. Results

3.1. eNOS and iNOS are located in brown adipocyte nuclei

In the animals kept at room temperature (22°C), brown adipocytes showed cytoplasmic staining with eNOS and iNOS antibodies. Interestingly, and unlike the other positive cell types in the tissue (endothelial cells of parenchymal capillaries and arteries, smooth muscle cells of large blood vessels and some nerves), some brown adipocytes also showed spe-

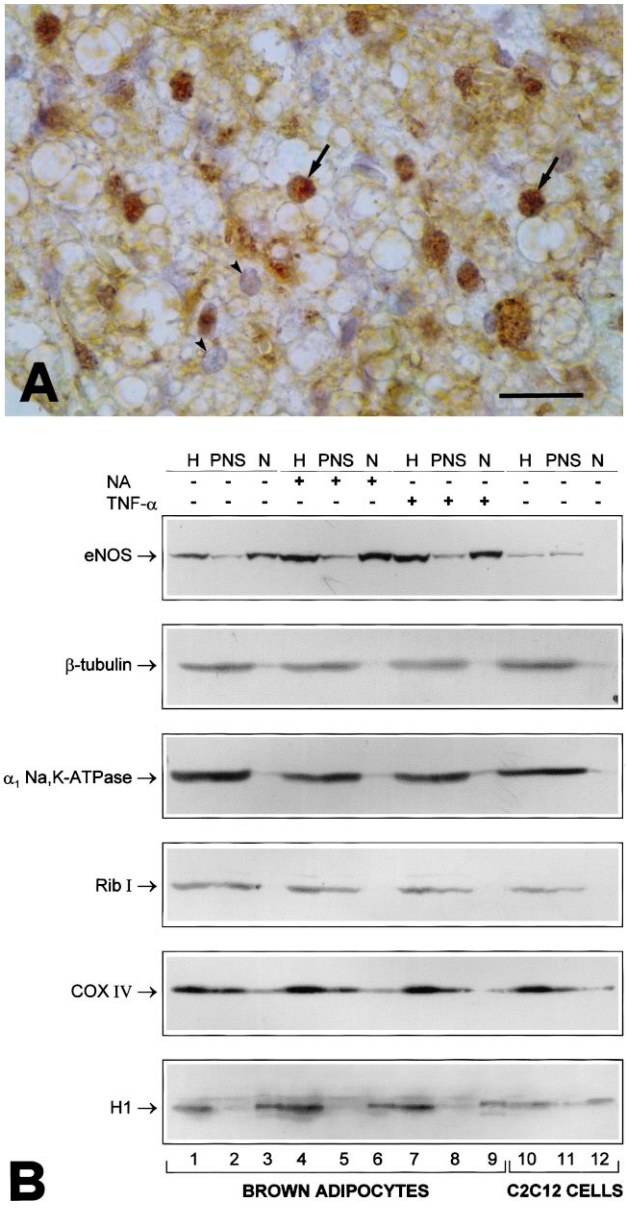

Fig. 1. A: BAT eNOS immunohistochemistry (polyclonal antibody, 1:250). In rats kept at room temperature, some brown adipocytes show specific nuclear staining (arrows). Negative nuclei are also indicated (arrowheads). Bar, 27 μm. B: Immunoblotting analysis of cell fractions obtained from cultured brown adipocytes and C2C12 cells (polyclonal antibody, 1:250). eNOS expression was studied in homogenate (H), postnuclear supernatant (PNS) and nuclear (N) fractions of cells treated or not (lanes 1–3 and 10–12) with NA (1 μM) or TNF-α (10 nM) (lanes 4–9).

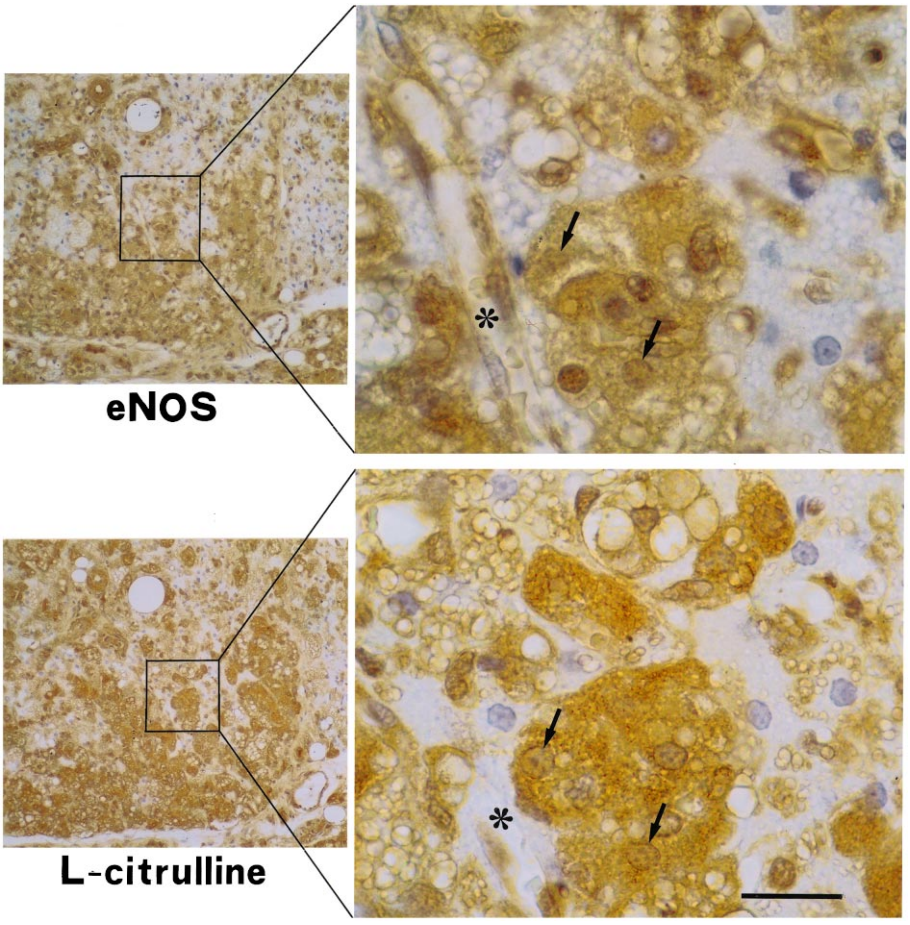

Fig. 2. eNOS (polyclonal antibody, 1:250) and L-citrulline (polyclonal antibody, 1:700) immunohistochemistry on BAT serial sections. At low magnification (left), the parenchymal fields positive for eNOS are also positive for L-citrulline. At higher magnification (right), both eNOS and L-citrulline are found in the same cells and nuclei (arrows). * Capillary. Bar, 130 μm (left); 20 μm (right).

cific nuclear staining for eNOS (Fig. 1A) and iNOS (not shown). At 22°C, weak cytoplasmic but no nuclear staining was observed with nNOS antibodies. Positive controls (hepatocytes and Kupffer cells of LPS-treated rats for iNOS, skeletal muscle and aortic endothelium for eNOS, and cerebellum granular layer neurons and glial cells for nNOS) exhibited the expected cytoplasm staining.

The nuclear localization of the eNOS isoform was further studied using subcellular fractionation, whereas cytosolic (postnuclear supernatant, PNS) and nuclear fractions were analyzed on immunoblot assays. The amount of protein in the pellet and supernatant fractions loaded on the gel was adjusted to correspond to the same number of cells. Both nuclear and PNS fractions contained a polypeptide with an apparent M_r of ~140 kDa corresponding to eNOS (Fig. 1B, lanes 1–3). Fraction purity was verified by Western blotting with antibodies raised against markers for PNS (β-tubulin), plasma membrane (Na,K-ATPase α_1 subunit), endoplasmic reticulum (Rib I), mitochondria (COX IV) and nucleus (H1) (Fig. 1B).

Immunoblotting analysis of the C2C12 cell line, where eNOS is expressed in large amount [19], showed that eNOS was totally concentrated in the PNS, but was not found in the nuclear fraction (Fig. 1B, lanes 10–12).

Both PNS and nuclear iNOS levels were below the detection threshold of Western blotting in unstimulated conditions. The nNOS immunoblot confirmed the immunohistochemical results, showing a very faint band in PNS but not in the nuclear fraction (not shown).

3.2. *eNOS and iNOS are functionally active in brown adipocyte nuclei*

To achieve the microscopic visualization of NOS catalytic activity [20], we performed the immunolocalization of L-citrulline. Blood vessels and brown adipocytes were diffusely stained, the latter in the cytoplasm and/or nuclei. Pretreatment of rats with L-NIL and L-NMMA, which specifically inhibit the activities of the inducible and endothelial NOS isoforms, remarkably reduced both cytoplasmic and nuclear staining for L-citrulline (not shown), thus also confirming the specificity of the antibody. Immunohistochemical analysis of serial sections showed that the brown adipocytes positive for L-citrulline were also positive for eNOS (Fig. 2) or iNOS in the cytoplasm and/or nucleus.

More direct evidence of the presence of the NOS isoforms in cytosol and nuclei was obtained by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in cell fractions from brown adipocytes differentiated in culture. As shown in Fig. 3A, more than 35% of the Ca^{2+}-dependent (measured in 10 μM $CaCl_2$-supplemented culture medium, without EGTA/EDTA) and 15% of the Ca^{2+}-independent (1 mM EGTA/EDTA, without $CaCl_2$) enzyme activities in control cells

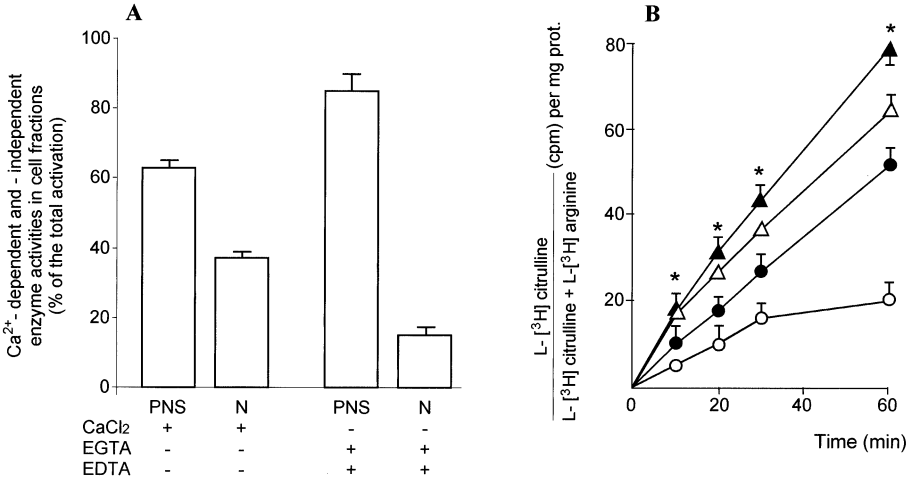

Fig. 3. Ca^{2+}-dependent and -independent NOS activities in cultured brown adipocytes. A: Percentage of NOS activities in postnuclear supernatant (PNS) and nuclear (N) fractions of untreated cells, as measured by conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. B: Conversion of radiolabeled L-arginine to L-citrulline in nuclear protein extracts from brown adipocytes differentiated in culture treated (▲, ●) or not (△, ○) with NA (1 μM) at different times and in the presence (△, ▲) or absence (○, ●) of calcium (10 μM $CaCl_2$, without EGTA/EDTA, or 1 mM EGTA/EDTA, without $CaCl_2$, respectively).

were found in the nuclear fraction, and were only partially lost in the absence of calcium. This strengthens the hypothesis that total nuclear NOS activity can be ascribed to both the Ca^{2+}-dependent (eNOS) (~69%) and the Ca^{2+}-independent (iNOS) (~31%) isoforms (Fig. 3B).

3.3. *The expression and functional activities of nuclear eNOS and iNOS are modulated by NA both in vivo and in vitro*

To gain further insights into the role of sympathetic activity in the in vivo modulation of nuclear eNOS and iNOS expression and functional activity in brown adipocytes, BAT from rats exposed to four different experimental conditions was subjected to immunohistochemical analysis. In animals exposed to 4°C for 3 or 14 days, eNOS and iNOS cytoplasmic staining was greater than in rats acclimated to room temperature. Accordingly, the number of positive nuclei increased progressively and peaked after 14 days (Table 1).

Cold-induced iNOS expression in rat BAT is mimicked by β_3-adrenoceptor stimulation of cultured brown adipocytes [10]. To test the specific involvement of β_3-adrenoceptors, lean rats acclimated at 22°C were treated for 1 week with CL 316,243 (1 mg/kg/day, i.p.), a selective thermogenically active β_3-adrenoceptor agonist [21]. Compared with saline-treated rats, these animals showed a significantly higher number of nuclei positive for eNOS and iNOS (Table 1).

Finally, NOS isoform expression and intracellular localization were analyzed immunohistochemically in BAT slices from genetically obese (fa/fa) rats, in which BAT sympathetic activity is markedly reduced [22,23]. In these animals, the number of iNOS-immunostained nuclei was significantly lower than in normal-weight (+/fa) rats (22 ± 3% vs. 43 ± 4%; Fig. 4), whereas the number of eNOS-positive nuclei was the same (47 ± 3% vs. 48 ± 4%).

Interestingly, immunostaining for L-citrulline (and for iNOS

Table 1
Percentage of iNOS- and eNOS-immunostained nuclei in brown fat of rats kept at room temperature (22°C) or acclimated to cold for 3 or 14 days, or treated with CL 316,243 (1 mg/kg/day, i.p.) for 1 week

	22°C	4°C (3 days)	4°C (14 days)	CL 316,243
iNOS	9 ± 1	21 ± 3*	43 ± 3*	54 ± 4*
eNOS	32 ± 3	30 ± 2	48 ± 3*	63 ± 2*

*$P < 0.005$ vs. untreated rats acclimated to room temperature.

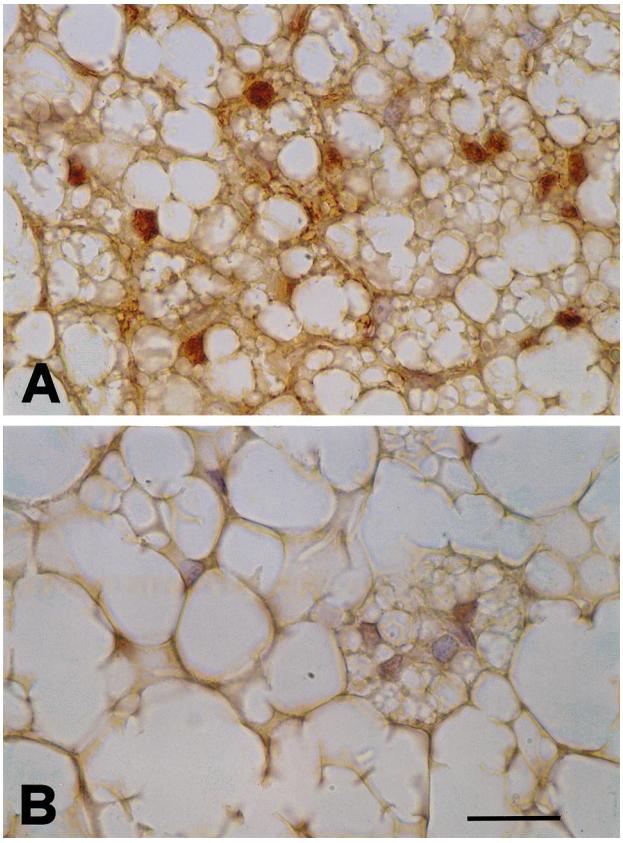

Fig. 4. BAT iNOS immunohistochemistry (polyclonal antibody, 1:500) in obese rats. In normal-weight (+/fa) rats (A), numerous brown adipocytes show nuclear staining; in obese (fa/fa) rats (B), few brown fat cells show a weak nuclear staining. Bar, 20 μm.

and eNOS) was more intense in the animals acclimated to cold for 3 or 14 days or treated with CL 316,243 than in those kept at room temperature (not shown), suggesting that, in addition to increasing their expression, noradrenergic stimulation enhances the functional activity of the two nuclear NOS isoforms. This finding was supported by in vitro experiments: the exposure of cultured brown adipocytes to 1 μM NA for 24 h increased the nuclear activities of both eNOS (10 μM $CaCl_2$, without EGTA/EDTA) (40% above the baseline) and iNOS (1 mM EGTA/EDTA, without $CaCl_2$) (35% above the baseline) as measured by conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (Fig. 3B).

Finally, Western blotting analyses showed that eNOS expression significantly increased in the PNS and nuclear fractions of the cells treated with NA (1 μM; 24 h) or TNF-α (10 nM; 24 h) compared with untreated cells (Fig. 1B, lanes 4–9).

4. Discussion

Brown adipocytes are endowed with NOS enzymes [10]. As it diffuses over the vascular bed, NO is likely involved in the cold-induced matching of BAT thermogenesis/vasodilation [9,10]. In addition, NO has been shown to improve the differentiation of cultured brown adipocytes [24] and to affect the respiratory rate of isolated BAT mitochondria [25] by an autocrine action. The present results show that eNOS and iNOS are located not only in the cytoplasm but also in the nucleus of brown adipocytes, where their amount and functional activities are dynamically modulated by noradrenaline. This entails that NO can be directly produced in the nucleus of these cells.

Interestingly, serial BAT sections from animals acclimated to cold for two different periods showed that both cytoplasmic and nuclear eNOS and iNOS are expressed, or more intensely expressed, in the same brown adipocytes that are positive, or more intensely positive, for UCP1 (unpublished observations). Thus, NOS expression in brown adipocytes is positively correlated with cellular activation and heat production.

We have recently reported that treatment of cultured brown fat cells with NO-generating *S*-nitroso-L-glutathione (300 μM; 60 min) increases the expression of the genes involved in brown fat cell differentiation and function, including peroxisome proliferator-activated receptor γ-2, UCP1 [24] and c-fos (unpublished data). Thus, a possible function of NO produced directly in the nucleus may be the modulation of gene expression. Indeed, an action of NO on gene expression has been well documented in several cell types, via cytoplasmic activation of transduction pathways such as cGMP [26] and calcium [27]. However, NO is an extremely reactive and unstable molecule in biological systems. Its steady-state concentration – on which its biological effects depend – is critically determined not only by its rate of production but also by its rate of decomposition, the latter being closely related to microenvironmental redox conditions [28]. Brown adipocytes are unique cells in this regard: when stimulated by NA, the rapid induction of UCP1 uncouples oxidative phosphorylation, with a concomitant increase in metabolic rate and fat utilization. Increased free radical generation due to raised fatty acid oxidation, relative hypoxemia, and heat production subject brown adipocytes to a metabolic stress that may create an environment 'hostile' to the action of NO. The localization or migration of the NOS isoforms into the nucleus might provide a subcellular environment more suitable than cytosol for a more specific and effective action of NO-producing systems, and for their close control. It would not be surprising if a similar control of NO signaling also intervened in other cell types under physiological or pathophysiological conditions. Of note, the presence of nuclear NOS has been demonstrated, albeit not conclusively, in cultured neonatal rat cardiomyocytes [29], glandular elements of normal and carcinomatous human endometrium [30], and pancreatic β-cells [31].

Finally, we have recently reported that the isoforms of heme oxygenase (HO) – a ubiquitous microsomal enzyme that produces carbon monoxide (CO), a novel gaseous mediator [32] – are detectable in both the nucleus and cytosol of rat brown adipocytes and that inducible HO-1 (but not constitutive HO-2) is up-regulated in the nucleus following cold exposure [33] and down-regulated in genetic obesity (unpublished data).

The present data, together with the research previously carried out by our group [10,24,33], suggest that brown adipocytes contain noradrenergically modulated nuclear enzyme systems able to produce biologically active gaseous mediators such as NO and CO, which could cooperate in controlling brown fat cell biology.

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