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Norepinephrine but not hypoxia stimulates HIF-1α gene expression in brown adipocytes ☆

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Abstract

The cellular response to hypoxic stress is mainly mediated via activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α). In the present study, the sympathetically controlled brown adipose tissue was used to investigate the effect of norepinephrine on HIF-1α gene expression. Norepinephrine increased HIF-1α mRNA levels in cultured brown adipocytes, whereas the hypoxia-mimic cobalt was without effect. Cold exposure of mice increased HIF-1α gene expression in brown adipose tissue. In UCP1-ablated mice, which are incapable of inducing thermogenic oxygen consumption in brown adipose tissue, cold exposure generated a significantly higher elevation of HIF-1α mRNA levels than in wild-type. These results demonstrate that cold-induced HIF-1α gene expression is independent of thermogenic oxygen consumption leading to hypoxia, but is consistent with a norepinephrine regulation of HIF-1α gene expression. Thus, by elevating HIF-1α gene expression, norepinephrine may mediate an increased potential to respond to hypoxia in brown adipose tissue and possibly in other tissues.
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Keywords: Norepinephrine; Hypoxia-inducible factor-1α; Hypoxia; Brown adipocytes; Brown adipose tissue; mRNA; Gene expression

The physiological response to generation of hypoxic oxygen levels in a tissue involves an increase in the expression of angiogenic factors, which generate a switch in the existing vasculature to an activated angiogenic state leading to increased tissue perfusion and oxygen delivery to the tissue. The primary mediator of the hypoxia-induced gene expression is the hypoxia-inducible factor-1 (HIF-1) (for reviews, see [1–4]). The active HIF-1 transcription factor is a heterodimer of HIF-1α and the aryl hydrocarbon receptor nuclear translocator. Regulation of the HIF-1 signalling pathway occurs at multiple levels, mainly via stabilisation of HIF-1α protein, regulation of HIF-1α translation, and regulation of transcriptional activity by recruitment of transcriptional co-activators. Regulation of this pathway may also involve stimulation of HIF-1α gene expression, but this level of regulation is relatively less studied.

In the present study, we have investigated a possible physiological regulation of the HIF-1 signalling pathway at the level of HIF-1α gene expression in brown adipose tissue. The function of brown adipose tissue is to generate heat to enable mammals to maintain body temperature at low ambient temperatures. During cold exposure of an animal, sympathetic nerves release the neurotransmitter norepinephrine in brown adipose tissue, which induces thermogenic processes through activation of the mitochondrial uncoupling protein UCP1 [5], and also cold-induced hyperplasia and hypertrophy [6,7]. The tissue hypertrophy also involves endothelial cell proliferation [8] and a marked increase in the capacity for blood perfusion [9,10], i.e., angiogenesis, leading to a tremendously dense vascularisation of the tissue [11]. Accordingly, inhibition of angiogenesis leads to a marked decrease in brown adipose tissue

☆ *Abbreviations:* HIF-1α, hypoxia-inducible factor-1α; UCP1, uncoupling protein-1; VEGF, vascular endothelial growth factor.

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growth [12]. The dense vascularisation of the tissue is required since the thermogenic process consumes very high amounts of oxygen during norepinephrine-activated thermogenesis. However, the high rate of oxygen consumption still leads to a near depletion of oxygen in the tissue [9].

In order to examine whether the norepinephrine-stimulated tissue hypertrophy also includes recruitment of an enhanced potential for defence against hypoxia, we investigated here the effect of norepinephrine on HIF-1α gene expression. Using cultured brown adipocytes, we demonstrated for the first time that norepinephrine is a direct stimulator of HIF-1α gene expression. Further, cold exposure led to elevated HIF-1α gene expression in brown adipose tissue, which was not dependent on generation of low oxygen levels by thermogenic oxygen consumption, but was consistent with an adrenergic regulation of expression. The present data thus suggest that norepinephrine, by elevating HIF-1α gene expression, may be involved in increasing the potential to respond to hypoxic conditions in brown adipose tissue and possibly in other tissues.

Materials and methods

Brown adipocyte primary cultures. Brown adipocyte precursors were isolated from the interscapular brown adipose tissue of 3-week-old mice of the NMRI strain or from the C57Bl/6 strain (B&K UNIVERSAL) or from UCP1-ablated mice [13] by the procedure described previously [14]. Cells were cultured in six-well plates in 2 ml of Dulbecco's modified Eagle's medium (Gibco-BRL without L-glutamine type), 4 mM L-glutamine added, supplemented with 10% newborn calf serum (PAN systems GmbH), 4 nM insulin (Actrapid Human, Novo), 10 mM Hepes (Gibco-BRL), and 50 IU penicillin, 50 μg streptomycin (Gibco-BRL), and 25 μg sodium ascorbate (Kebo) per ml. The cells were incubated at 37 °C in an 8% CO_2 atmosphere. The medium was changed every other day until the experimental day (i.e., day 6) when cells were treated or not with norepinephrine ((−)-Arterenol bitartrate; Sigma) or cobalt chloride.

Animals and cold exposure. Female 7-week-old C57Bl/6 mice (B&K UNIVERSAL) and UCP1-ablated mice [13] were housed for 7 days in plastic cages in rooms at 30 °C with a 12-h light–dark cycle and given free access to laboratory chow and water. On the experimental day, animals were transferred to single cages at 4 °C for 1–4 h, after which time the animals were killed and tissues were collected and frozen in liquid nitrogen. The experiment was repeated four times with one animal per time point and genotype.

RNA isolation and Northern blotting. Total RNA was extracted from brown adipose tissue and from cultured cells using Ultraspec RNA Isolation System (Biotecx Laboratories, Texas, USA), according to the manufacturer's instructions. After quantification by UV absorbance at 260 nm, 20 μg RNA from tissue samples and 10 μg RNA from cell culture samples were separated on a 1% agarose/formaldehyde gel and transferred to a Hybond N membrane (Amersham Pharmacia Biotech), and mRNA levels were quantified as described previously [14,15]. Equal loading of the gel and the integrity of RNA were routinely verified by observing the ethidium bromide-stained gel under UV-light.

cDNA probes. The HIF-1α cDNA was a gift from Dr. Georg Breier, Max-Planck Institute, Bad Neuheim, Germany. It contained a full-length murine HIF-1α cDNA cloned into a pcDNA3 vector. The vector was amplified by transformation of a TG-1 *E. coli* strain, and the cDNA was isolated. The UCP1 cDNA and β-actin cDNA were those used earlier [16]. Each cDNA probe was labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) by Ready To Go DNA Labeling Beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Western blotting. Cultured cells were lysed, including sonication, and total protein was separated on an 8% SDS–PAGE gel, followed by electroblotting as previously described [17]. Membranes were incubated overnight at 4 °C with a monoclonal anti-HIF-1α antibody (Calbiochem) and the primary antibody was detected as previously described [17].

Statistical analysis. Statistical analyses were made with paired or unpaired *t* test as described in the figure legends. The level for statistical significance is indicated with asterisks in the figures as follows: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; (*): $p < 0.1$. Hatches indicate statistical difference between genotypes.

Results

Effect of norepinephrine on HIF-1α gene expression

To investigate the effect of norepinephrine on HIF-1α gene expression, we used brown adipocytes cultured for 6 days to give visually well-differentiated cells. When HIF-1α gene expression was analysed, we observed a strong expression of the gene in the cultured brown adipocytes (Fig. 1A, lanes 1 and 3), which has not previously been demonstrated.

The ability to express the UCP1 gene is a characteristic of differentiated brown adipocytes [5]. Therefore, as a positive control, we analysed UCP1 gene expression after treating the cells with norepinephrine, which is the physiological inducer of the UCP1 gene. As expected, norepinephrine induced a massive increase in UCP1 gene expression (Fig. 1B). These results verify that the cultured brown adipocytes had indeed differentiated and that the norepinephrine stimulation was fully effective.

Norepinephrine stimulation significantly increased HIF-1α mRNA levels to about 50% above control levels (Figs. 1A and C). This result adds norepinephrine to the few factors demonstrated to stimulate HIF-1α gene expression.

Effect of hypoxia-mimic and norepinephrine on HIF-1α mRNA and protein levels in brown adipocytes

Hypoxia, being the most recognised activator of the HIF-1 pathway, has been demonstrated to have stimulatory effect on HIF-1α gene expression in some cell types [18,19], but to lack effect in other cell types [20,21]. Therefore, we investigated whether hypoxia had an effect on HIF-1α gene expression in brown adipocytes. Brown adipocyte cultures were treated with the hypoxia-mimic cobalt chloride. We have earlier shown that cobalt treatment has a strong effect on VEGF gene expression in these brown adipocytes, with about a 3-fold elevation of VEGF mRNA after 6 h [14]. However, although norepinephrine had a highly significant stimulatory effect on HIF-1α gene expression in all cell cultures studied, we could not detect any significant effect of cobalt treatment (Fig. 2A).

Hypoxia-induced activation of the HIF-1 signalling pathway is mainly mediated via stabilisation of HIF-1α protein, which has a very short half-life under normoxic conditions and is virtually undetectable in normoxic cells [1–4]. We could verify the elevation of HIF-1α protein

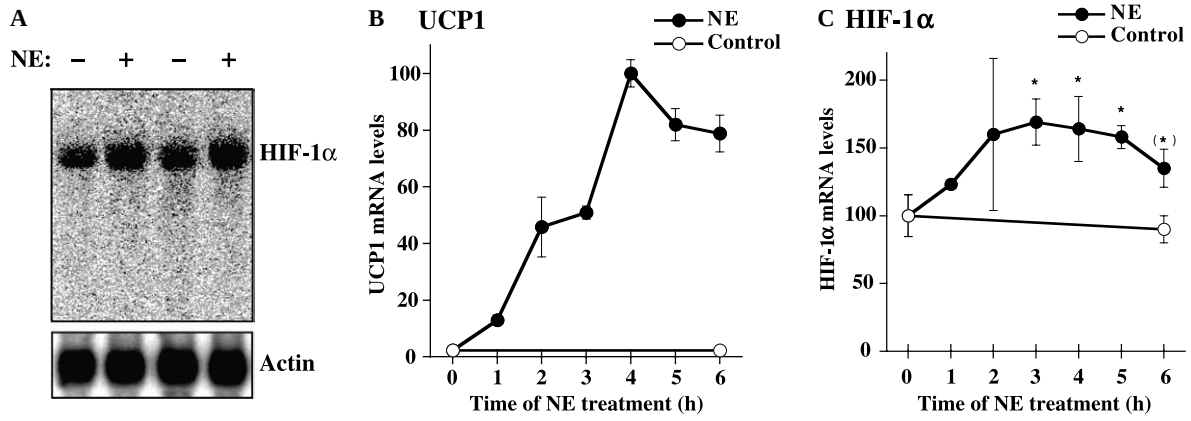

Fig. 1. Norepinephrine stimulation of HIF-1α gene expression. Brown adipocytes were isolated from NMRI mice, cultured for 6 days as described under Materials and methods, and treated as indicated. Total RNA was isolated and used for Northern blotting. (A) The upper panel is a Northern blot of HIF-1α mRNA and the lower panel shows β-actin mRNA to verify equal loading. Cells were treated with 10 μM norepinephrine (NE) for 1 h as indicated. (B) UCP1 mRNA levels after treatment with 10 μM norepinephrine for 0–6 h. Values are means ± SE from one experiment with triplicate wells for each time of treatment. The mean value for 4 h was set to 100%. (C) HIF-1α mRNA levels in the same experiment as in (B). The mean value for 0 h control was set to 100%. Asterisks indicate the level of statistical difference versus 0 h control with unpaired *t* test.

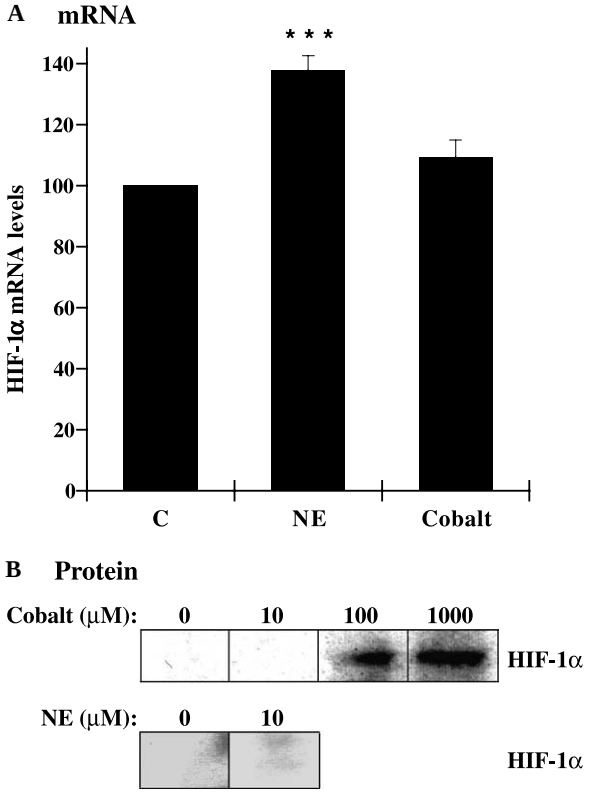

Fig. 2. Effect of hypoxia-mimic on HIF-1α mRNA and protein levels in brown adipocytes. Brown adipocytes were isolated from NMRI mice, cultured for 6 days as described under Materials and methods and treated as indicated. (A) Cells were treated with 10 μM norepinephrine (NE) for 1 h or 100 μM cobalt chloride (Cobalt) for 6 h, after which total RNA was isolated and HIF-1α mRNA levels were determined as in Fig. 1. C, untreated control. Values are means ± SE of five experiments with 2–4 wells for each treatment. Control levels were set to 100% in each experimental series. Asterisks indicate statistical difference versus control with paired *t* test. (B) Cells were harvested and cell extracts were used for Western blotting as described under Materials and methods. The panel shows HIF-1α protein levels after 5 h treatment with the indicated concentrations of cobalt chloride (Cobalt) and norepinephrine (NE).

levels with cobalt treatment (Fig. 2B), which has not previously been demonstrated in brown adipocytes. We investigated whether the norepinephrine-induced elevation of HIF-1α mRNA resulted in increased HIF-1α protein levels. However, treatment of brown adipocytes with norepinephrine over several hours generated no detectable levels of HIF-1α protein (Fig. 2B).

Effect of cold exposure on HIF-1α gene expression in brown adipose tissue

To investigate whether the norepinephrine effect on HIF-1α gene expression in vitro has any physiological relevance, we cold-exposed mice and analysed HIF-1α gene expression in brown adipose tissue. Cold exposure leads to a marked increase in sympathetic activity in brown adipose tissue in situ and drives recruitment of the tissue in order to meet the increased demand for non-shivering thermogenesis in the animal [5]. Cold exposure generated about a 2-fold increase in HIF-1α mRNA levels in brown adipose tissue (Fig. 3A). The kinetics were similar to the effect of norepinephrine on cultured brown adipocytes (Fig. 1C).

Significance of thermogenic oxygen consumption in cold-induced HIF-1α gene expression

To investigate whether hypoxic conditions in the tissue, generated by the oxygen consuming thermogenic process [5,9], mediated the cold-induced effect or if it was a direct effect of norepinephrine, we additionally analysed the effect of cold exposure on HIF-1α gene expression in brown adipose tissue of UCP1-ablated mice. The lack of UCP1 leads to a complete lack of norepinephrine-induced oxygen consumption in isolated brown adipocytes [22], and the UCP1-ablated animals have no capacity for cold-induced brown adipose tissue thermogenesis and oxygen consumption

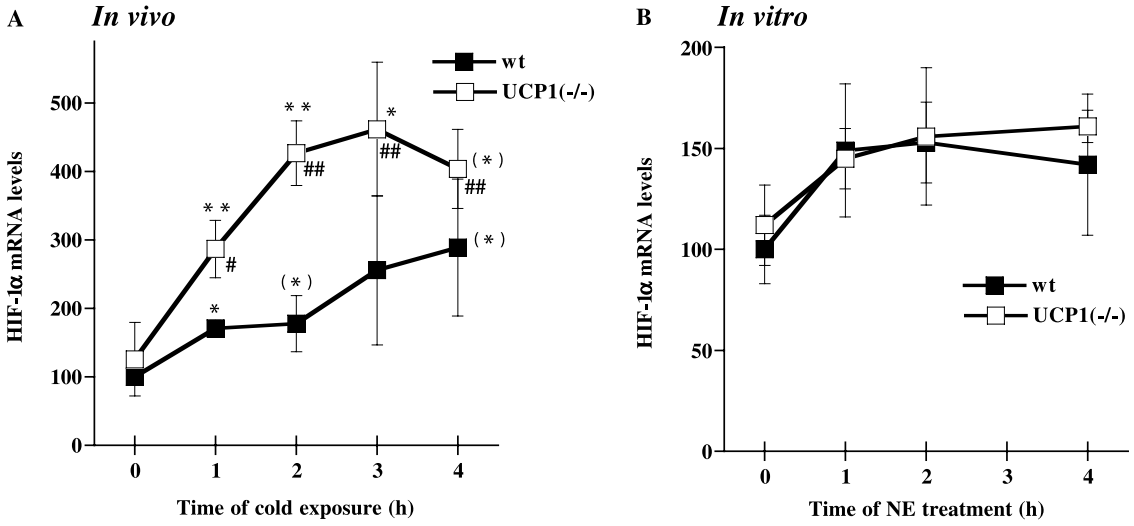

Fig. 3. Cold exposure stimulates HIF-1α gene expression in brown adipose tissue. (A) Animals were cold exposed at 4 °C for the times indicated, after which the animals were killed and interscapular brown adipose tissue was dissected out. Total RNA was isolated and HIF-1α mRNA levels were determined by Northern blot analysis as described under Materials and methods. Filled squares, C57Bl/6 wild-type mice (wt); open squares, UCP1-ablated mice (UCP1(−/−)). Values are means ± SE from four experiments. The value for wt 0 h was set to 100% in each experimental series. Asterisks indicate statistical difference versus the start value (0 h) and hatches indicate statistical difference between genotypes for each time point with paired *t* test. (B) Brown adipocytes were isolated and cultured for 6 days as described under Materials and methods. Cells were treated with 10 μM norepinephrine (NE) for the indicated times, after which HIF-1α mRNA levels were determined as in Fig. 1. Filled squares, cells from C57Bl/6 wild-type mice (wt); open squares, cells from UCP1-ablated mice (UCP1(−/−)). Values are means ± SE from one experiment with triplicate or quadruplicate wells for each time of treatment and with eight wells for 0 h controls. The mean value for wt 0 h controls was set to 100%. There were no statistical differences between genotypes.

[13,23]. If cold-induced HIF-1α gene expression is secondary to hypoxia, no increase in expression would be expected in these mice. However, in the UCP1-ablated mice, cold exposure had a significantly stronger stimulatory effect on HIF-1α gene expression than in wild-type animals (Fig. 3A). These results indicate that hypoxic conditions generated in the brown adipose tissue by the thermogenic process are not involved in the cold-induced HIF-1α gene expression, in agreement with the lack of effect of cobalt treatment on cultured brown adipocytes (Fig. 2A).

The higher increase in brown adipose tissue HIF-1α gene expression in UCP1-ablated mice could be due to an increased responsiveness to norepinephrine in the brown adipocytes of the UCP1-ablated mice. To investigate this possibility, we cultured brown adipocyte precursors from both genotypes. Norepinephrine stimulated HIF-1α gene expression in C57Bl/6 wild-type cells (Fig. 3B), in agreement with the data from the NMRI mice brown adipocyte cultures (Figs. 1 and 2A). In brown adipocytes from UCP1-ablated animals, norepinephrine also stimulated HIF-1α gene expression (Fig. 3B). However, in contrast to the in vivo data (Fig. 3A), brown adipocytes from UCP1-ablated mice demonstrated no enhanced responsiveness to norepinephrine stimulation, but demonstrated basal and norepinephrine-induced levels identical to wild-type cells (Fig. 3B). Thus, these results demonstrate that UCP1 ablation does not alter the norepinephrine responsiveness of the HIF-1α gene and is not the explanation for the higher induction seen in vivo (Fig. 3A).

Norepinephrine levels in brown adipose tissue from both wild-type and UCP1-ablated mice were recently analysed in our laboratory. UCP1-ablated mice had 2.5-fold higher norepinephrine levels, a more than 3-fold higher norepinephrine turnover rate, and thus an estimated 8-fold higher sympathetic activity in brown adipose tissue compared to wild-type (Golozoubova et al., unpublished results). Apparently, the absence of a thermogenic response from brown adipose tissue results in a lack of inhibitory feedback regulation of sympathetic norepinephrine release, and thus leads to hyperactive sympathetic activity and elevated norepinephrine levels in the tissue. Thus, the markedly higher sympathetic activity observed in UCP1-ablated mice may be the explanation for the stronger effect on HIF-1α gene expression in these animals (Fig. 3A).

Discussion

In the present investigation, we demonstrate for the first time that the neurotransmitter norepinephrine is a stimulator of HIF-1α gene expression, at least in brown adipocytes. Cold exposure increased HIF-1α gene expression in brown adipose tissue, which was independent of increased oxygen consumption in the tissue. Hormonal regulation may also explain an elevated HIF-1α gene expression in other tissues under hypoxic stress.

Stimulation of HIF-1α gene expression in cell culture

Over the past several years, numerous reports have elucidated the mechanisms of activation of the HIF-1 signalling pathway, and it is well established that inhibition of HIF-1α proteosomal degradation and regulation of

transcriptional activity are the primary regulatory mechanisms of this pathway in activation of target gene expression [1–4]. In comparison, only a few reports have demonstrated regulation of HIF-1α gene expression as a potential mechanism in regulation of the HIF-1 signalling pathway. An increase in HIF-1α mRNA may not in itself lead to an increased amount of the HIF-1 transcription factor, provided that proteosomal degradation is adequately high. However, if HIF-1α mRNA levels are elevated, a given decrease in degradation activity could result in higher amounts of HIF-1α protein. Thus, elevation of HIF-1α mRNA may be said to increase the potential for defence against hypoxia.

Hypoxia in itself has been reported to have stimulatory effects on HIF-1α gene expression in a few cell types [18,19], but appears to lack this effect in most cell types [20,21,24,25]. We found that also in brown adipocytes hypoxia was without effect (Fig. 2A).

A few non-hypoxic factors have until now been demonstrated to affect HIF-1α gene expression in certain cell types. In cartilage chondrocytes and fibroblasts, interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide stimulate HIF-1α gene expression [26,27], and angiotensin II in vascular smooth muscle cells [20]. In melanocytes and melanoma cells, HIF-1α gene expression is stimulated via a α-melanocyte stimulating hormone/cAMP/microphthalmia-associated transcription factor pathway [38]. Interferon-α treatment may down-regulate HIF-1α gene expression [28].

Thus, whereas the stabilisation of HIF-1α protein by hypoxia appears to be a universal phenomenon, stimulation of HIF-1α gene expression by hypoxia and non-hypoxic factors is apparently highly cell specific, and norepinephrine is here demonstrated to be a stimulator of HIF-1α gene expression in brown adipocytes.

Physiological control of elevation of HIF-1α gene expression

Although hypoxic stimulation of HIF-1α gene expression in isolated cells is only found in a few cell types, systemic hypoxia has been demonstrated to result in elevation of HIF-1α mRNA levels in rodent brain, lung, kidney, and heart tissues [29–32]; in human cardiac tissue, HIF-1α mRNA levels were higher in ischemic areas than in non-ischemic areas [33,34]. Similarly, skeletal muscle has also indications for involvement of elevated HIF-1α gene expression in adaptation to hypoxic conditions in the tissue. Skeletal muscle HIF-1α mRNA levels increased in subjects training in low oxygen levels [35], although hypoxia-mimic does not increase HIF-1α mRNA in muscle cells [25]. Further, in a rodent model for bone fracture repair where regional hypoxia develops, HIF-1α gene expression was markedly elevated [36].

These results suggest that although the cells may not in themselves be able to respond to hypoxia by increased HIF-1α gene expression, elevated HIF-1α gene expression is involved in the response of several tissues to hypoxia, but the increase is mediated by an agent, the nature of which is not known.

In the present study, we demonstrate that generation of hypoxic conditions by high oxygen consumption in brown adipose tissue is not a necessary factor for cold-induced HIF-1α gene expression in the tissue, in accordance with the lack of effect of hypoxia-mimic on cultured brown adipocytes (Fig. 2A). Rather, the present demonstration of a direct stimulatory effect by norepinephrine on HIF-1α gene expression in brown adipocytes (Figs. 1 and 2A), and the high cold-induced HIF-1α expression levels in brown adipose tissue of UCP1-ablated mice (Fig. 3A), parallelled by a markedly elevated sympathetic activity in the tissue, imply mediation by norepinephrine rather than by hypoxia itself.

In parallel, it may be noted that systemic hypoxia leads to increased myocardial norepinephrine levels [37], implying a possible mediation by norepinephrine in this tissue, similarly to what we found in brown adipose tissue. Thus, norepinephrine may be an inducer of HIF-1α gene expression in several cell types.

Conclusion

Several recent reports, together with the data in the present study, indicate that a physiological upregulation of HIF-1α gene expression may be part of the functional preparation of a tissue to hypoxic conditions. The present data indicate that norepinephrine, not hypoxia, is the mediator of the elevated HIF-1α gene expression in brown adipose tissue, by a direct effect on the brown adipocytes. Also in other tissues under sympathetic control, it is possible that norepinephrine, and not hypoxia itself, is the mediator of elevated HIF-1α gene expression. Further studies are required to determine the involvement of norepinephrine in elevation of HIF-1α gene expression in the adaptation process to hypoxic conditions in other tissues such as brain, heart, and skeletal muscle.

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