Norepinephrine Induces Vascular Endothelial Growth Factor Gene Expression in Brown Adipocytes through a β-Adrenoreceptor/cAMP/Protein Kinase A Pathway Involving Src but Independently of Erk1/2*

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To identify the signaling pathway that mediates the adrenergic stimulation of the expression of the gene for vascular endothelial growth factor (VEGF) during physiologically induced angiogenesis, we examined mouse brown adipocytes in primary culture. The endogenous adrenergic neurotransmitter norepinephrine (NE) induced VEGF expression 3-fold, in a dose- and time-dependent manner (EC50 ~ 90 nM). Also, the hypoxia-mimicking agent cobalt, as well as serum and phorbol ester, induced VEGF expression, but the effect of NE was additive to each of these factors, implying that a separate signaling mechanism for the NE-mediated induction was activated. The NE effect was abolished by propranolol and mimicked by isoprenaline or BRL-37344 and was activated. The NE effect was abolished by propranolol and mimicked by isoprenaline or BRL-37344 and was activated. The NE-induced VEGF expression was fully cAMP mediated, an effect which was inhibited by H-89 and thus was dependent on protein kinase A activity. Involvement of other adrenergic signaling pathways (a1-adrenoreceptors, Cu²⁺, protein kinase C, a2-adrenoreceptors, and pertussis toxin-sensitive G1-proteins) was excluded. The specific inhibitor of Src tyrosine kinases, PP2, markedly reduced the stimulation by NE, which demonstrates that a cAMP-dependent Src-mediated pathway is positively connected to VEGF expression. However, inhibition of Erk1/2 MAP kinases by PD98059 was without effect. NE did not prolong VEGF mRNA half-life and its effect was thus transcriptional, and was independent of protein synthesis. These results demonstrate that adrenergic stimulation, through β-adrenoreceptor/cAMP/protein kinase A signaling, recruits a pathway that branches off from the NE-activated Src-Erk1/2 cascade to enhance transcription of the VEGF gene.

For the study of angiogenesis, brown adipose tissue provides a physiologically interesting model system. In this tissue, norepinephrine (NE),1 the adrenergic mediator in sympathetic nerves, is the acute inducer of thermogenesis (1). During the thermogenic process, the rate of lipid oxidation in this tissue, and thus the demand for oxygen delivery to the tissue, is immense. Heat production in brown adipose tissue accounts for nearly half of the total energy expenditure in a cold-acclimated animal in the cold, even though the tissue only constitutes 1–2% of the total body weight (2, 3). The blood supply needed to support the oxygen requirement under such conditions is necessarily exceedingly high (2, 3), demanding a rich vasculature, which is indeed increased during brown adipose tissue recruitment (2, 4).

The regulation of this angiogenesis in brown adipose tissue has so far not been extensively investigated. Expression of the angiogenic factor basic fibroblast growth factor, is induced in the tissue by cold exposure (5) and by NE in cultured brown adipocytes (6). The VEGF-related factor (vascular endothelial growth factor-B) is expressed already during development (7), and expression of VEGF (vascular endothelial growth factor (8)) itself is induced by NE administration and cold exposure in brown adipose tissue (9), and VEGF expression and secretion is stimulated by NE in cultured brown adipocytes (10, 11). Thus, NE appears to mediate cold-induced angiogenesis in brown adipose tissue by stimulating the expression of angiogenic factors, such as VEGF. In this respect, the effect of NE may be connected to the general recruitment effect of NE in this tissue, i.e., increased DNA synthesis (6, 12–14) and increased expression of the brown adipocyte-specific mitochondrial uncoupling protein UCP1 (15–18).

The aim of the present investigation was to clarify through which intracellular signaling mechanisms NE stimulates expression of the VEGF gene. We conclude that NE-induced VEGF expression was exclusively dependent on a β-adrenoreceptor-mediated increase in cAMP levels and PKA activity. The mediation involved Src tyrosine kinases, unexpectedly and completely independently of downstream mediation via Erk1/2 MAP kinases. We thus demonstrate a positive connection between VEGF expression and a signaling pathway that branches off from the NE-activated Src-Erk1/2 cascade.

EXPERIMENTAL PROCEDURES

Cell Isolation—Brown adipocyte precursors were isolated from 3–4-week-old mice of the NMRI strain, obtained from a local supplier (Eklunds), principally as described by Néchad et al. (19) and modified by Bronnikov et al. (14). Briefly, the cervical, interscapular, and axillary brown adipose tissue depots were dissected out from each mouse. The depots were pooled and incubated in the Hepes-buffered Ringer solution detailed by Néchad et al. (19), containing 0.2% (w/v) crude collagenase type II (Sigma). The tissue was digested for 30 min at 37 °C, with vortexing every 5 min, after which the digest was filtered through a 250-μm nylon screen. To allow lipid droplets and mature adipocytes to float, the digest was put on ice for 20 min, and the infranatant was then collected and filtered through a 25-μm nylon screen. The cells in the
Briefly, proteins were separated on 12% polyacrylamide gels. Electrophoresis was carried out with the Cyclic AMP Assay System (Amersham Pharmacia Biotech), and aliquot from each sample was used for determination of cAMP levels in a Heraeus CO2 auto-zero B5061 incubator. On day 1, the cultures were treated the cultures with NE for 1 h. VEGF mRNA levels increased approximately 3-fold after NE stimulation (Fig. 1A, top arrow), which is in accordance with observations made in cultured rat brown adipocytes (11). The stimulation by NE was observed at all stages of brown adipocyte differentiation, from proliferating to fully differentiated cells, and was of similar relative magnitude (Fig. 1C, black bars).

When brown adipocytes were stimulated with increasing concentrations of NE, VEGF mRNA levels increased monophasically after 5 min follow by quenching (5% (v/v) calf serum), and with 0.5% (w/v) albumin (fraction V, fatty acid free; Roche Molecular Biochemicals) added. The experiments were performed after approximately 20 h in the medium.

Results

Mouse Brown Adipocytes Constitutively Express the VEGF Gene

To examine the regulation of VEGF expression in mouse brown adipocytes, we first investigated the ability of these cells to express the VEGF gene constitutively. Total RNA isolated from differentiated mouse brown adipocytes (on day 6 in culture) was analyzed by Northern blot analysis. The 580-base pair mouse VEGF cDNA probe (21) was used. On the Northern blot (Fig. 1A), a major band at approximately 4 kilobases was observed (top arrow), and 4 further bands at lower molecular weight were distinguishable on densitometric analysis. This Northern blot pattern was similar to that observed in other cells, tissues, and species (9, 21, 23, 24), including rat brown adipose tissue (9). During the different treatments used in this study, the intensity of these bands varied in parallel, and the total signal was therefore that used for analysis.

To investigate whether the expression of VEGF is a differentiation-dependent change, we followed VEGF expression during cell differentiation. Brown adipocytes in primary culture proliferate until confluence is reached, which occurs at approximately day 5–6 after inoculation (14, 19, 20, 25, 26). At this time, differentiation spontaneously occurs. This is verified in Fig. 1B where the ability of NE to induce expression of the brown adipocyte-specific differentiation marker, mitochondrial uncoupling protein-1 (UCP1), is analyzed. As seen, rather high levels of UCP1 mRNA were induced by NE from day 5 (Fig. 1B, black bars), indicating that brown adipocyte differentiation had occurred. As seen in Fig. 1C (gray bars), VEGF was constitutively expressed already in undifferentiated cells, and basal VEGF mRNA levels were similar in all stages of differentiation. This is in contrast to VEGF expression observed in 3T3-F442A adipocytes, which was fully differentiation-dependent (27). Thus, the state of differentiation is not crucial for basal VEGF expression in brown adipocytes.

Norepinephrine Induces VEGF Gene Expression

To investigate the effect of NE on VEGF expression, we treated the cultures with NE for 1 h. VEGF mRNA levels increased approximately 3-fold after NE stimulation (Fig. 1A, black bar), which is in accordance with observations made in cultured rat brown adipocytes (11). The stimulation by NE was observed at all stages of brown adipocyte differentiation, from proliferating to fully differentiated cells, and was of similar relative magnitude (Fig. 1C, black bars).

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RNA Isolation and Determination of mRNA Levels for VEGF and UCP1—On the indicated day of culture (day 6 if not otherwise indicated) and after stimulation, the culture medium was discarded and the cells were dissolved in 0.8 ml of an Ultraspec solution (Biotex), and the manufacturer’s procedure for RNA isolation was followed. The RNA concentration was measured on a Beckman DU 50 spectrophotometer with readings at 260 and 280 nm; the 260/280 nm ratio was routinely seen to be in the range (18). The probes were labeled with .

cDNA Probes—The VEGF cDNA was a gift from Dr. Georg Breier, Max-Planck Institute, Bad Nauheim, Germany. It contained the 580-base pair mouse VEGF164 cDNA, which encodes the VEGF 164 isoform (21). This cDNA had been cloned into the Escherichia coli strain and the cDNA was isolated. The UCP1 cDNA was that earlier used (18). The probes were labeled with .

cAMP Analysis—On day 6 in culture and after stimulation, the culture medium was aspirated as the indicated time and the cells were treated principally as described by Bronnikov et al. (20). Briefly, 0.8 ml of 75% ethanol with 1 ml EDTA was added to each well, and after 10 min, cells were harvested by scraping. Ethanol was removed by SpeedVac centrifugation and pellets were suspended in 0.5 ml of 4 M guanidinium thiocyanate (buffer) and sonicated 5 s. After centrifugation, a 25-μl supernatant from each sample was used for determination of cAMP levels with the Cyclic AMP Assay System (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Erk1/2 Phosphorylation Analysis—On day 6 in culture and after stimulation, the culture medium was discarded and the cells were harvested and analyzed for Erk1/2 phosphorylation as described earlier (22). Briefly, proteins were separated by electrophoresis using the ImageQuant program. The total signal was therefore that used for analysis.

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sically, following simple Michaelis-Menten kinetics, to 4-fold control levels, obtained with 10 μM NE; the EC50 was 70 ± 22 nM (Fig. 2A). Analysis of mean points from four similar experiments yielded a 3-fold increase, with similar kinetics; EC50 93 ± 27 nM (not shown).

In the maintained presence of NE, the VEGF mRNA levels were elevated only during the first few hours and had returned to control levels after 6 h (Fig. 2B). An independent experiment with 30 min NE stimulation of brown adipocytes resulted in only a 30% elevation of VEGF mRNA levels (not shown); thus maximum levels occurred at about 1 h of stimulation. The rapid disappearance of the mRNA signal in these experiments implies a short half-life of VEGF mRNA (see also below). This transient increase in expression is in principal agreement with the time course of VEGF expression induced in rat brown adipose tissue in situ by cold exposure: the expression peaked at 4 h, and had decreased down to control levels within 24 h (9).

Based on these experiments, further experiments were performed with 10 μM NE and analyzed after 1 h of stimulation.

Interaction between Norepinephrine and Other Inducers of VEGF Gene Expression

As demonstrated above, NE is a potent inducer of VEGF expression in mouse brown adipocytes. In various other cell types, other factors such as hypoxia, cobalt (as a hypoxia-mimicking agent), serum, growth factors, and phorbol esters have been demonstrated to induce VEGF expression (27–31). We have therefore investigated whether NE is an exclusive inducer of VEGF expression in brown adipocytes, or whether the classical factors mentioned above could induce expression also in these cells. These experiments may also reveal initial information on the intracellular pathway mediating the NE

![Fig. 2. Dose and time response curves for norepinephrine-induced VEGF gene expression. Brown adipocytes were isolated and cultured for 6 days, and stimulated as indicated, and VEGF mRNA levels were analyzed principally as described in the legend to Fig. 1A. A, VEGF mRNA levels after 1 h stimulation with the indicated concentrations of NE. The values are mean ± S.E. of quadruplicate wells from one experiment. The mean control value was set to 100%. Curve was drawn according to simple Michaelis-Menten kinetics, yielding an EC50 of 70 ± 22 nM, with a maximal level of 432 ± 21% of control. B, time course for NE-stimulated VEGF expression. 10 μM NE was added at time 0. The points are mean ± S.E. of three experiments with single wells. Zero time values were set to 100% in each experiment. •, NE treated; ○, untreated controls.](http://www.jbc.org/)**Fig. 2.**
signal, as non-additivity between the NE-induced VEGF expression and that induced by other factors would imply distinct signaling pathways.

Hyperoxia (Cobalt)—The hyperoxia-mimicking agent cobalt was not able to elevate VEGF expression after 1 h of treatment (not shown), but after 6 h, a 3-fold increase could be seen (Fig. 3A). The elevated VEGF mRNA levels persisted for at least 24 h (not shown). Thus, hyperoxia is probably an inducer of VEGF expression also in brown adipocytes. The effect of NE was additive to that of cobalt (Fig. 3A). Thus, NE and hyperoxia (cobalt treatment) utilize separate pathways for the induction of VEGF expression.

Serum—To investigate the effect of serum factors, serum was removed from the brown adipocyte cultures. When readded the next day, serum induced VEGF expression 4-fold (Fig. 3B). The effect of serum lasted longer than that of NE (≥6 h; not shown). NE was able to induce VEGF expression, also in the absence of serum factors (Fig. 3B). The effect of NE was additive to that of serum (Fig. 3B), and thus the signaling pathway mediating the NE effect would also seem to be different from pathways utilized by serum factors.

Phorbol Esters—The phorbol ester TPA was also a potent inducer of VEGF expression (4-fold) (Fig. 3C), implying that protein kinase C (PKC)-activated pathways can stimulate VEGF expression in brown adipocytes. NE gave a marked increase of VEGF mRNA in addition to that induced by TPA alone (Fig. 3C). Thus, the pathway mediating the NE effect on VEGF expression appears not to involve PKC activation (see also below). Thus, also the classical inducers of VEGF expression, hyperoxia (cobalt), serum, and phorbol ester, induced VEGF expression in brown adipocytes, but NE utilized a distinct signaling pathway.

Mediation of Norepinephrine-induced VEGF Gene Expression

To investigate which adrenergic receptors mediate the NE effect on VEGF expression in brown adipocytes, adrenoreceptor agonists and antagonists were used (Fig. 4A). The Ø-1-adrenoreceptor antagonist prazosin had no significant effect on NE-induced VEGF expression, but the Ø-2-adrenoreceptor antagonist propranolol completely abolished it. In accordance with this, the Ø-1-adrenoreceptor agonist cirazoline had no inducing effect (Fig. 4A), nor had the Ø-3-adrenoreceptor agonists clonidine (10 μM) or UK14304 (10 μM) (not shown), whereas the Ø-2-adrenoreceptor agonist isoprenaline was an equally potent inducer as was NE (Fig. 4C). Thus, only Ø-2-adrenoreceptors mediated the NE-induced VEGF expression.

Proliferating brown preadipocytes only express the Ø-2-adrenoreceptor subtype whereas fully differentiated brown adipocytes also express the Ø-3-adrenoreceptor subtype which, at this stage of differentiation, is the only Ø-2-adrenoreceptor subtype coupled to adenylyl cyclase (20). Thus, the NE-induced VEGF expression observed in proliferating undifferentiated brown preadipocytes (days 3–4, Fig. 1B) is most likely mediated by Ø-2-adrenoreceptors. In differentiated brown adipocytes, the Ø-3-adrenoreceptor agonist BRL-37344 induced VEGF expression to the same extent as did NE (not shown). The Ø-2-adrenoreceptor agonist CGP-12177 (32) (which is a Ø-1- and Ø-2-adrenoreceptor antagonist) also induced VEGF expression in these cells (not shown). Thus, both Ø-1- and Ø-3-adrenoreceptors were able to mediate NE-induced VEGF expression in these cells, the coupling depending on the differentiation stage. To identify which second messengers had the ability to induce VEGF expression, we examined the intracellular second messenger systems involved in adrenoreceptor-mediated signaling: Ca2+- and PKC (from Ø-1-adrenoreceptors), the proposed Ø-2-adrenorecep-

![Fig. 3. Interactions between norepinephrine and classical inducers of VEGF gene expression.](http://www.jbc.org/) VEGF expression was analyzed in brown adipocytes principally as described in the legend to Fig. 1A. A, brown adipocytes were stimulated for 6 h with 100 μM cobalt chloride (Co); controls (C) received 100 μM magnesium chloride. After 5 h, some cultures received 10 μM NE. After 6 days in culture, the medium was discarded and replaced with the serum-free medium detailed under “Experimental Procedures.” After 24 h, newborn calf serum (S) was added as indicated to a concentration of 10%, with or without 10 μM NE. The cultures were harvested 1 h later. The values are mean ± S.E. of four experiments with duplicate wells for each treatment. Mean control values (C) were set to 100% in each experimental series; p < 0.001 (two-way ANOVA with replicates) comparing cobalt to cobalt + NE. B, after 6 days in culture, the medium was discarded and replaced with the serum-free medium detailed under “Experimental Procedures.” After 24 h, newborn calf serum (S) was added as indicated to a concentration of 10%, with or without 10 μM NE. The cultures were harvested 1 h later. The values are mean ± S.E. of four experiments with duplicate wells for each treatment. Mean control values (C) were set to 100%; p < 0.001 (two-way ANOVA with replicates) comparing serum to serum + NE. C, brown adipocytes were treated for 1 h with either 500 ng/ml TPA (T) or vehicle (Me2SO), with or without 10 μM NE. Values are mean ± S.E. from one experiment with quadruplicate wells for each treatment. Mean control value (C) was set to 100%; p < 0.001 (one-way ANOVA) comparing TPA to TPA + NE.
pretreatment, followed by 1 h acute 500 ng/ml TPA (Fig. 4B) and/or 10 μM TPA pretreatment, 20 h for each treatment. Mean control values were set to 100%.

B, NE. The values are mean VEGF mRNA levels after 20 h with 500 ng/ml TPA or vehicle (Me2SO).

abolished (Fig. 4B), and the acute effect of TPA on VEGF mRNA levels was remaining elevation of VEGF mRNA levels was observed (Fig. 4D). Thus, increased levels of cAMP are potent stimulators of VEGF expression in brown adipocytes and may mediate the NE effect.

cAMP Is the Mediator of the Norepinephrine-induced VEGF Expression

To establish that cAMP not only is able to induce VEGF expression, but also is the actual mediator of the NE effect, we first investigated the correlation between cAMP levels and VEGF expression in brown adipocytes treated with different doses of forskolin. The elevation of cAMP levels was dose-dependent (Fig. 5A), as was the elevation of VEGF mRNA levels (Fig. 5B). However, nearly maximal induction of VEGF expression occurred already at approximately 50 pmol of cAMP/well (Fig. 5C). Thus, the ability of cAMP to induce VEGF expression was saturated at this cAMP level.

To compare the ability of NE- and forskolin-derived cAMP to induce VEGF expression, we measured the cAMP levels induced by NE (Fig. 5D). The NE-induced cAMP levels reached the saturating levels for VEGF expression (approximately 50 pmol of cAMP/well), and the dose-response kinetics were very similar to those for NE-induced VEGF expression (Fig. 2A). Indeed, when NE-derived results were plotted into the forskolin curve (Fig. 5E), they did not deviate from the forskolin results. These results suggest that NE-induced VEGF expression is exclusively dependent on cAMP. To confirm this, we treated brown adipocytes with NE at forskolin concentrations sufficient to saturate cAMP-induced VEGF expression. Under these conditions, NE did not affect forskolin-induced cAMP levels (not shown), nor did it affect forskolin-induced VEGF expression (Fig. 5F). This lack of additivity implies that cAMP is the common mediator of NE- and forskolin-induced VEGF expression (contrast the additivities in Fig. 3).

The Signaling Pathway Downstream of cAMP Is Mediated via PKA and Src but Not via Erk1/2

To identify the further signaling pathway involved in mediating the CAMP-dependent stimulation of VEGF expression, we used inhibitors of intracellular signaling factors.

The classical immediate downstream effector of cAMP, the cAMP-dependent protein kinase (PKA), could be expected to mediate the CAMP-dependent signaling. Therefore, we pretreated brown adipocytes with the PKA inhibitor H-89, which

not altered by TPA pretreatment (Fig. 4B) and was thus not mediated through a TPA-sensitive PKC pathway.

To investigate the possible involvement of the suggested CAMP-independent β2-adrenoreceptor signaling through pertussis toxin (PTX)-sensitive G-proteins, i.e., Gi-proteins (33, 37, 38), we pretreated brown adipocytes with PTX. PTX augmented β2-adrenoreceptor-stimulated CAMP production,2 confirming that Gi proteins were active. However, NE- or isoprenaline-induced VEGF expression was not affected by PTX, and PTX had no effect in itself (Fig. 4C). Thus, no adrenergic stimulation of VEGF expression was mediated through PTX-sensitive Gi proteins in a CAMP-independent process.

To investigate the ability of cAMP to induce VEGF expression, brown adipocytes were treated with forskolin to activate adenyl cyclases. Forskolin induced VEGF expression to the same extent as did NE (Fig. 4A). This effect was not altered by increased levels of intracellular Ca2+ (Fig. 4A). The forskolin analogue 1,9-dideoxy-forskolin, which does not activate adenyl cyclases, had no effect (not shown). The CAMP analogue 8-bromo-cAMP (1 mM for 1 h) also induced VEGF expression 3-fold (not shown). Thus, increased levels of cAMP are potent stimulators of VEGF expression in brown adipocytes and may mediate the NE effect.

...tor/Gi-protein signaling pathway (33), and cAMP (from β-adrenoreceptors).

The Ca2+ ionophore A23187, which increases intracellular Ca2+ levels and through this, e.g., induces c-fos expression (34), had no effect on VEGF mRNA levels (Fig. 4A). Concerning PKC activation, it was shown above that the phorbol ester TPA induced a marked elevation of VEGF expression (Fig. 3C), but it was concluded that the NE effect appeared to be independent of PKC, because of the additivity of the effects. To substantiate this, PKC was inactivated prior to NE stimulation, by prolonged exposure to TPA, a treatment that inactivates TPA-sensitive PKC isoforms (35, 36). After TPA pretreatment, no remaining elevation of VEGF mRNA levels was observed (Fig. 4B), and the acute effect of TPA on VEGF mRNA levels was abolished (Fig. 4B). Thus, TPA pretreatment effectively inactivated PKC. However, the NE-induced VEGF expression was...
had no effect in itself, but which abolished both NE- and forskolin-induced VEGF expression (Fig. 6). That also the forskolin effect was inhibited demonstrated that H-89 acted by inhibiting PKA, and not by competing with NE for binding (such an effect has been demonstrated for β1- and β2-adrenoceptors (39), but does not seem to apply to β3-adrenoceptors).3 Thus, the cAMP-dependent stimulation was mediated through a PKA-dependent signaling pathway.

In brown adipocytes, adrenergic activation may proceed via Src tyrosine kinases, which are activated by β3-adrenoceptors in a PKA-dependent manner in these cells.4 Therefore, to investigate whether Src mediates NE-induced VEGF expression, we pretreated brown adipocytes with the specific inhibitor of Src, PP2. PP2 was fully effective as a Src inhibitor, since NE-induced Erk1/2 phosphorylation was abolished (Fig. 7A). To ensure that PP2 did not have a specific effect on adrenergic pathways and gene expression, we first examined whether PP2 inhibited NE-induced UCP1 expression. Clearly PP2 did not have a general inhibitory effect; rather, surprisingly, PP2 markedly potentiated the effect of NE (Fig. 7B), an effect for which we have no explanation. Concerning the NE-induced VEGF expression, PP2 had a marked inhibitory effect (Fig. 7C). Thus, Src was involved in the mediation of the response, although a Src-independent pathway also existed.

The MAP kinases Erk1/2 could be expected to mediate the Src-dependent stimulation of VEGF expression. This cascade is activated by NE in brown adipocytes in a cAMP-dependent manner (22, 40). In other cell types, VEGF expression (induced by other factors) has been implied to be induced via this signaling cascade (24, 30, 41–43). Therefore, we pretreated brown adipocytes with the MAP kinase/Erk1/2 kinase (MEK1) inhibitor PD98059. In agreement with earlier observations (22), PD98059 totally abolished NE-induced Erk1/2 phosphorylation (Fig. 8A). However, PD98059 did not alter the NE-induced VEGF expression (Fig. 8B). Thus, despite the involvement of Src demonstrated above, Erk1/2 did not mediate NE-induced VEGF expression in brown adipocytes. In an attempt to characterize factors acting downstream of Src tyrosine kinases on the stimulation of VEGF expression, we used the general tyrosine kinase inhibitor, genistein (100 μM, 1 h pretreatment), but it decreased NE-induced VEGF expression to a lesser extent than did PP2 (not shown).

Phosphatidylinositol 3-kinase is a substrate for Src (44) and is a factor implicated in the activation of VEGF expression (45). However, wortmannin (100 nm, 1 h pretreatment), an inhibitor of phosphatidylinositol 3-kinase, had no effect on NE-induced VEGF expression (not shown). Thus, phosphatidylinositol 3-kinase-dependent signaling pathways did not appear to be involved.

Norepinephrine Does Not Require Induced Protein Synthesis to Induce VEGF Gene Expression

To investigate whether synthesis of new transcription factors, or other protein factors, were required for NE-induced
VEGF expression, we examined the effect of the translational inhibitor cycloheximide. Cycloheximide in itself led to an induction of VEGF mRNA (Fig. 9). A similar effect has been described in other systems (46, 47). These experiments imply either that a short-lived protein factor may constitutively inhibit VEGF transcription or, perhaps more likely, that such a factor may promote VEGF mRNA degradation (in other systems, cycloheximide has been demonstrated to stabilize VEGF mRNA (48)).

The effect of NE was additive to that of cycloheximide (Fig. 9) and thus not dependent on protein synthesis. Thus, although NE may induce the gene expression of certain transcription factors (such as hypoxia-inducible factor-1α),5 such induction cannot be involved in the mediation of the NE effect. The lack of need for protein synthesis is also in accordance with observations in other systems, with other inducers of VEGF expression (46, 49). Thus, all factors required for regulation of VEGF expression appeared to be constitutively produced and present in brown adipocytes.

**Fig. 7.** Effect of the Src inhibitor PP2 on norepinephrine-induced Erk1/2 phosphorylation and UCP1 and VEGF gene expression. Brown adipocytes were cultured for 5 days after which the cells were cultured under serum-free conditions for 20 h (to decrease basal Erk1/2 phosphorylation (22)) as described under “Experimental Procedures,” and pretreated with 50 μM PP2 or vehicle (Me2SO) for 1 h, followed by stimulation as indicated. A, Erk1/2 phosphorylation (analyzed as detailed under “Experimental Procedures”) after stimulation with 10 μM NE for 5 min as indicated. The values are mean ± S.E. of three experiments with duplicate wells. Mean NE values were set to 100%. B, UCP1 mRNA levels after 1 h with 10 μM NE. The values are mean ± S.E. of three experiments with duplicate wells for each treatment in parallel cultures to those in A. Mean NE values were set to 100%. C, VEGF mRNA levels after 1 h with 10 μM NE. The values are mean ± S.E. of three experiments with duplicate wells for each treatment in parallel cultures to those in A. Mean NE values were set to 100%. (Also in three similar experiments in normal (serum-containing) medium, PP2 inhibited NE-induced VEGF expression; p < 0.001 (two-way ANOVA with replicates).)

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5 J. M. Fredriksson, H. Nikami, and J. Nedergaard, unpublished observations.
The Effect of Norepinephrine on VEGF Gene Expression Is Transcriptional

The augmenting effect of NE on VEGF mRNA levels could be due to an increase in the rate of transcription of the VEGF gene or to an increase of VEGF mRNA half-life. Indeed, in several cell types, the half-life of VEGF mRNA is positively affected by other agents, such as hypoxia (50, 51), PKC activation (48), and nitric oxide (52). To investigate whether NE functions by prolonging VEGF mRNA half-life, we treated brown adipocytes with the transcriptional inhibitor actinomycin D. The VEGF mRNA half-life, as measured after actinomycin D treatment, was approximately 1.5 h (Fig. 10) (compare also the rate of spontaneous decrease seen in Fig. 2B), which is similar to earlier observations (51, 53). NE did not affect the half-life of VEGF mRNA (Fig. 10). Thus, the effect of NE was probably due to a direct increase in the rate of transcription.

DISCUSSION

In the present investigation, we have demonstrated that the adrenergic agent NE induces the expression of the gene for the potent angiogenic factor VEGF in primary cultures of mouse brown adipocytes. The stimulatory effect by NE occurred independently of that induced by other factors known to induce VEGF expression in other cell systems. Signaling was via a β-adrenoreceptor-induced increase in cAMP levels, further mediated by PKA. Downstream of cAMP/PKA, the signal was mediated by Src tyrosine kinases and also through a Src-independent pathway. However, the Src pathway did not involve Erk1/2 MAP kinases (Fig. 11).

Induced synthesis of protein factors was not required for mediation of the adrenergic stimulation. The mechanism of action was not prolongation of VEGF mRNA half-life, and was thus apparently an increase in the rate of transcription.

Norepinephrine Stimulation of VEGF Gene Expression—That adrenergic stimulation of cultured rat (11) and mouse brown adipocytes stimulated VEGF expression, indicates paracrine/localization of the NE-induced expression observed in intact animals by Asano et al. (9). Although NE is not generally recognized as an inducer of VEGF expression, the effect is not exclusive for brown adipocytes, since also in rat aortic smooth muscle cells, adrenergic stimulation induces VEGF expression (54).

Adrenergic stimulation of VEGF expression in brown adipose tissue is physiologically meaningful in that also the oxygen-demanding thermogenic processes are adrenergically stim-

The Effect of Norepinephrine on VEGF Gene Expression was demonstrated here in cultured brown adipocytes, where elevated VEGF mRNA levels were observed only for the first few hours during chronic stimulation, may be thought to imply that adrenergic stimulation of VEGF expression may not be of major significance in regulating physiological angiogenesis. VEGF
expression induced by the hypoxia-mimicking agent cobalt was maintained for a longer period of time, and thus could be considered more physiologically relevant, as hypoxic conditions may be generated in brown adipose tissue in the cold-exposed animal, due to the extensive oxygen consumption by the adrenergic thermogenic processes (2, 3). However, in brown adipose tissue in cold-exposed rats (9) and mice, induced VEGF expression is also transient and thus, in this respect, very similar to our observations in NE-stimulated cultured brown adipocytes. Thus, it is likely that NE is the physiological inducer of VEGF expression and thereby of cold-induced angiogenesis. Apparently, an initial short elevation of VEGF expression is sufficient to support angiogenesis during brown adipose tissue recruitment.

β-Adrenergoreceptors, cAMP, PKA, and Src but Not Erk1/2 Mediate the Norepinephrine-induced VEGF Gene Expression

The classical β-adrenergoreceptor pathway, cAMP and PKA, was fully responsible for the mediation of the NE effect. In several cell types, forskolin and/or CAMP-analouges have earlier been demonstrated to induce VEGF expression (27, 46, 54–62). In most of these cell types, as yet only an ability of CAMP to induce VEGF expression has been demonstrated, but it has been implied that the relevant physiological activator also in these cells induces VEGF expression via cAMP. Further mediation has, however, not been asssigned.

It is in this context especially noteworthy that in brown adipocytes, Src tyrosine kinases are activated via a β3-adrenoceptor/cAMP/PKA pathway. This is of relevance because Src has numerous substrates involved in various cellular processes (44), but the factors mediating the adrenergic stimulation of VEGF expression downstream of Src are presently unknown. As the effect of NE does not involve prolongation of VEGF mRNA half-life, an increase in the rate of transcription of the VEGF gene is to be expected. Furthermore, as the NE induction of VEGF expression is not dependent of protein synthesis, the transcription factor involved must be constitutively expressed. The Src-dependent NE-induced VEGF expression may involve the transcription factor Sp1, which can mediate Src-dependent gene expression (65) and PDG-induced VEGF expression (66). Also NFκB may mediate Src-dependent gene expression (44) and thus may be involved. For these transcription factors, putative binding sites (consensus sequences) exist in the VEGF promoter (67, 68).

In conclusion, we have here demonstrated that adrenergic stimulation of VEGF expression in brown adipocytes is exclusively mediated by a β-adrenergoreceptor/cAMP/PKA signaling pathway. This pathway can utilize a Src-dependent pathway that branches off from the Src-Erk1/2 signaling cascade to stimulate expression of the VEGF gene.

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