Iodide deficiency-induced angiogenic stimulus in the thyroid occurs via HIF- and ROS-dependent VEGF-A secretion from thyrocytes

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In this study, we investigated the contributions of hypoxia-inducible factor (HIF) and reactive oxygen species (ROS) to the relationship between changes in thyrocyte iodide concentrations and VEGF release.

MATERIALS AND METHODS

Cell cultures. PCCL3 cells were a gift from Dr. F. Miot, (IRBBH, Brussels, Belgium), and human thyroids from multinodular goiters were obtained at surgery, after anatomopathologist examination, and after patients gave their informed consent. Cells were cultured as described previously (32). PCCL3 cells were grown to 80–90% confluence and human thyrocytes for 7–9 days in medium containing NaI at a concentration of 10−8 M in a humidified atmosphere (5% CO₂). The day of the experiment, culture media were replaced by fresh media with NaI (controls) or without NaI. Biological samples were then harvested after 1, 2, 4, or 6 h corresponding to the experimental times. Therefore, control cells were compared with cells facing an acute withdrawal of iodide. To make sure that changes in endpoints (ROS, HIF, and VEGF levels) were actually due to the acute withdrawal of iodide and not just the absence of iodide in the culture medium, a control experiment was carried out where thyrocytes incubated without iodide from the beginning of the culture (long-term deprivation) were compared with thyrocytes incubated with iodide. The role played by the acute drop in intracellular iodide contents was further addressed in an experiment where NaI-incubated cells were treated with NaClO₄ (perchlorate, 1 mmol/l) to block the sodium iodide symporter (NIS) instead of being deprived of iodide.

To verify the specificity of the role played by HIF-1 and ROS, cells were treated with either echinomycin (100 nmol/l; Alexis Biochemicals), a substance that inhibits the binding of HIF-1α to the hypoxia response element (HRE) located on the VEGF-A gene (21), or N-acetyl-cysteine (NAC; 1 mmol/l), a potent antioxidant, 1 h before medium replacement and in fresh medium with or without NaI. This study was approved by the Ethics Commitee of the Universite Catholique de Louvain.

127I distribution on semithin sections. Cells were harvested, centrifuged, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1.5 h. Each pellet was included in agar and embedded in LX112 resin (Ladd Research Industries, Burlington, VT). The elemental distribution of phosphorus and iodide (31P and 127I) was obtained by NanoSIMS50 imaging (7, 17, 29, 30). Maps (mean surface of 30 × 30 μm²) were acquired under standard analytical conditions using a Cs⁺ primary beam with impact energy of 16 keV and a current of 1 pA. Under these conditions, a lateral resolution of 100 nm is expected. Both elements were detected in a multicollection mode with parallel detection of carbon, nitrogen, and sulfur (12C, 12C14N, and 34S). All pictures were acquired in 256 × 256 pixels, with a counting time of 20 ms/pixel.

Quantitative PCR. For each condition, cells from five individual wells were suspended in TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany). Total RNA purification and reverse transcription were performed as described previously (14). cDNAs (2 μl) were mixed with 500 nM of each selected primer (see Table 1) and SYBR Green reaction mix (Bio-Rad) in a final volume of 25 μl.

THE TIGHT CONTROL OF THE MICROCIRCULATION is strictly required for all organs to safeguard oxygen and nutrient supplies. In endocrine organs, especially in the thyroid gland, the local microvasculature exerts additional roles that contribute to keep endocrine functions steady. One of the key elements of thyroid hormone synthesis is iodine, an essential trace element that is found chiefly in the environment. It must be provided continuously to maintain thyroid function. To achieve this goal, thyrocytes have developed various feedback systems, including rapid adaptations of the local vascular supply to changes in iodide delivery (10, 37, 40). Thyrocytes are tightly associated with capillaries in so-called angiofollicular units (12, 13, 15), and there is an inverse relationship between the rate of iodide supply and the local rate of angiogenesis (27). In this way, thyrocytes are able to react within hours to a reduction in intracellular iodine concentration by releasing angiogenic signals such as VEGF-A. Importantly, this rapid adaptation occurs in the absence of stimulation by thyroid stimulating hormone (TSH), the main trophic factor of the thyroid gland (11, 14, 23).

One of the most intriguing questions that has remained unanswered for years is how, in the absence of TSH stimulation, thyrocytes identify and translate the drop in intracellular stores of iodine, generate angiogenic signals acting on adjacent capillaries in so-called angiofollicular units (12, 13, 15), and there is an inverse relationship between the rate of iodide supply and the local rate of angiogenesis (27). In this paper is how thyrocytes, facing an acute drop in intracellular thyroid function, angiogenesis; vascular endothelial growth factor A; oxidative stress; hypoxia-inducible factor-1-dependent pathway.

Address for reprint requests and other correspondence: A.-C. Gérard, Unité de Morphologie Expérimentale, Université Catholique de Louvain, UCL-5251, 52 Av. E. Mounier, B-1200, Brussels, Belgium (e-mail: anne-catherine.gerard @uclouvain.be).

Am J Physiol Endocrinol Metab 296: E1414–E1422, 2009. First published March 31, 2009; doi:10.1152/ajpendo.90876.2008.—Vascular supply is an obvious requirement for all organs. In addition to oxygen and nutrients, blood flow also transports essential trace elements. Iodine, which is a key element in thyroid hormone synthesis, is one of them. An obvious requirement for all organs to safeguard oxygen and nutrient supplies. In this study, we investigated the contributions of hypoxia-inducible factor (HIF) and reactive oxygen species (ROS) to the relationship between changes in thyrocyte iodide concentrations and VEGF release.

Iodide deficiency-induced angiogenic stimulus in the thyroid occurs via HIF- and ROS-dependent VEGF-A secretion from thyrocytes. Am J Physiol Endocrinol Metab 296: E1414–E1422, 2009. First published March 31, 2009; doi:10.1152/ajpendo.90876.2008.—Vascular supply is an obvious requirement for all organs. In addition to oxygen and nutrients, blood flow also transports essential trace elements. Iodine, which is a key element in thyroid hormone synthesis, is one of them. An obvious requirement for all organs to safeguard oxygen and nutrient supplies. In
Reactions were performed in an iCycler (IQ5) from Bio-Rad as follows: 95°C/1.5 min, followed by 40 cycles of 95°C/15 s, annealing temperature (see Table 1)/45 s, and 81°C/15 s. Amplification levels were normalized to those of H9252-actin.

Western blotting. For VEGF-A protein detection, thyrocytes from five individual wells were suspended in Laemmli buffer (50 mM Tris/H18528 HCl, pH 6.8, 2% SDS, 10% glycerol) containing inhibitor cocktail (Sigma) and were sonicated for 30 s. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (20/H9262 g/lane) were heated at 95°C for 5 min in the loading buffer (Laemmli buffer containing 100 mM DTT and 0.1% bromophenol blue), separated by 10% SDS-PAGE electrophoresis, and transferred onto nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Rosendaal, The Netherlands).

For HIF-1α detection, thyrocytes were suspended in 70 µl of lysis buffer (50 mM imidazole, 300 mM KCl, 10 mM NaF, 1 mM EDTA, 0.5 mM MgCl2, 10 mM d-glycerophosphate, 1 mM Na3VO4, 1 mM DTT, 0.1 mM PMSF, and 1 mM benzamidine, pH 7) and immediately frozen in liquid nitrogen. Seventy microliters of 2/L1003 Laemmli buffer was then added. The loading buffer (3 µl of Laemmli containing 100 mM DTT and 0.1% bromophenol blue) was added to 35 µl of the protein solution. Proteins were separated by 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h in phosphate-buffered saline (PBS; pH 7.4), 5% nonfat dry milk, and 0.1% Tween and incubated overnight at 4°C with a polyclonal antibody raised against VEGF-A (1/100, VEGF A-20 for human cells and VEGF C-1 and VG-1 for PCCL3 cells; Santa Cruz Biotechnology) or HIF-1α (1/250 for PCCL3 cells (R & D Systems, Minneapolis, MN) and 1/250 for human thyrocytes (BD Biosciences, Franklin Lakes, NJ)). Membranes were washed with PBS-Tween 0.1%, incubated for 1 h at RT with EnVision (1/200; DakoCytomation, Carpinteria, CA) peroxidase-labeled antibody, and detected using enhanced chemiluminescence (ECL; Amersham Biosciences). Samples were analyzed using a Fuji Multigauge or Kodak Image Station 4000 MR (Eastman Kodak Co., Rochester, NY). The results were compared by computerized densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

Table 1. Primer sequences and annealing temperatures for qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>5’GATCCTGCGTCGCTGACCT3’</td>
<td>5’AGGAGGAGGATATTTTCTTGT3’</td>
<td>62°C</td>
</tr>
<tr>
<td>rVEGF</td>
<td>5’GAGTATATCTCCTGAGCCTGCT3’</td>
<td>5’TGTTCGTCGGTTTATCTGT3’</td>
<td>65°C</td>
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<tr>
<td>hVEGF</td>
<td>5’GGACACCTGCCGCTTTCGAAGAACAGA3’</td>
<td>5’GGAGCCGCCTCTCTTCTTAC3’</td>
<td>60°C</td>
</tr>
<tr>
<td>hNIS</td>
<td>5’CGCCCCTGGTTCTCGGCTGAG3’</td>
<td>5’ACGGCGCCTGGTTCACCT3’</td>
<td>59°C</td>
</tr>
<tr>
<td>rNIS</td>
<td>5’GGGCGTGGACTCTCCCGACTGAC3’</td>
<td>5’GGAGGCCCCTGACATGC3’</td>
<td>63°C</td>
</tr>
<tr>
<td>rHIF-1α</td>
<td>5’GGGGGTTTGGTATTCGGTAT3’</td>
<td>5’GGAGGCCCCTGACATGC3’</td>
<td>55°C</td>
</tr>
</tbody>
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qRT-PCR, quantitative RT-PCR; r, rat; h, human; NIS, sodium iodide symporter; HIF-1α, hypoxia-inducible factor-1α.

Reactions were performed in an iCycler (IQ5) from Bio-Rad as follows: 95°C/1.5 min, followed by 40 cycles of 95°C/15 s, annealing temperature (see Table 1)/45 s, and 81°C/15 s. Amplification levels were normalized to those of β-actin.

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Fig. 1. Iodide deprivation decreases intracellular 127I contents. 127I and 31P signals were localized using NanoSIMS in human thyroid cells incubated with or without iodide for 4 h. 31P detection indicates the presence of cell nuclei. 127I was detected as gray points in the cell cytoplasm of control cells incubated with iodide. This intracellular signal was gone in cells incubated without iodide for 4 h. Cells are delineated with a white line.
secondary antibody, washed, and visualized with enhanced chemiluminescence (SuperSignal West Femto; Pierce) on CL-Xposure films (Pierce). The same membranes were also treated with an anti-β-actin antibody (1/8,000; Sigma). Cells treated with the hypoxia-mimetic agent cobalt chloride (CoCl₂, 1 μM) were used as positive control.

**ELISA.** The release of VEGF-A by thyrocytes was assessed by ELISA using a specific commercially available kit specific for human or rat VEGF (Quantikine; R & D Systems). The quantity of total HIF-1α protein was measured on cellular extracts of human thyrocytes using a commercially available kit (DuoSet, Human/Mouse total HIF-1α ELISA; R & D Systems). The iron chelator and hypoxia-mimetic agent deferoxamine (DFO; 100 μM), which is known to induce HIF-1α activity, was used as positive control.

**HIF-1α immunocytochemistry.** Human thyrocytes were cultured in multichamber glass slides (Nunc International) for 9 days in medium containing NaI 10⁻⁸ M. The media were then replaced by fresh media containing NaI or no NaI for 2 h. Thyrocytes were fixed for 30 min in 4% paraformaldehyde, rinsed once with PBS, permeabilized for 1 h with Triton (1%) in PBS supplemented with 1% bovine serum albumin (PBS-BSA), and washed with PBS-BSA. Cells were then incubated with an anti-HIF-1α primary antibody (1/250 in PBS-BSA + 1% Triton; R & D Systems) for 1.5 h. After washing in PBS-BSA, fluorescein isothiocyanate-conjugated secondary antibody (1/30; Dako) was added for 1 h. Nuclei were stained by incubating cells with Hoechst for 30 min. Slides were mounted in fluorescent mounting medium (Dako) for microscopic observation. Negative control included omission of the primary antibody, and positive control is cells incubated for 1 h with CoCl₂ (1 μM) before immunofluorescence procedure.

**HIF-1α activity assay.** The activity of HIF-1α was measured on nuclear extracts by using an active HIF-1α activity assay kit (DuoSet, human/mouse active HIF-1α activity assay; R&D Systems) according to the manufacturer’s protocol. Briefly, a biotinylated double-stranded (ds) oligonucleotide containing a consensus HIF-1α-binding site was incubated with nuclear extracts. HIF-1α ds oligonucleotide complexes were subsequently captured by an immobilized antibody specific for HIF-1α. After unbound material was washed away, detection using streptavidin-horseradish peroxidase was performed. The specificity of the assay was tested using an unlabelled ds competitor oligonucleotide. DFO-treated cells were used as positive controls.

**ROS production by human thyrocytes.** Human thyrocytes were incubated in multichamber glass slides for 9 days in medium containing NaI 10⁻⁸ M. The media were then replaced by fresh media containing NaI or no NaI for 1 or 2 h. ROS production was measured using a fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Paisley, UK). Cells were washed with PBS and then incubated in Krebs-Ringer HEPES (pH 7.4) containing...
DCFH-DA (25 μM) for 1 h at 37°C, 5% CO2. The excess of dye was removed by two washes with PBS, and slides were mounted in fluorescent mounting medium (Dako). ROS production was visualized on a fluorescent microscope equipped with a digital camera (Zeiss).

Statistical analysis. For PCCL3 cells, all experiments were repeated at least twice. Data are expressed as means ± SE of five independent wells (n = 5) of one representative experiment. For VEGF-A quantitative (q)PCR in human thyrocytes, results are expressed as means ± SE of mean values of five experiments. Western blots were scanned and quantified by densitometry using the NIH Scion Image Analysis Software (National Institutes of Health, Bethesda, MD). Western blots and qPCR data were normalized with β-actin. The iodide-deficient groups were compared with controls using unpaired t-test. P < 0.05 was considered statistically significant. For ELISA tests, statistical analysis were performed using ANOVA followed by a Tukey-Kramer or Newman-Keuls multiple comparison post hoc tests (GraphPad InStat, San Diego, CA).

RESULTS

Iodide deprivation increases VEGF-A expression and release. Basal VEGF-A mRNA expression was first evaluated in cells incubated with or without iodide from the beginning of the culture (long-term deprivation) to make sure that observa-
Hence, a rapid drop in intracellular I contents occurs in the cytoplasm of cells incubated without iodide for 4 h (Fig. 1).

Iodide deprivation induced a 1.7-fold increase in VEGF-A mRNA expression (P < 0.05) in human thyrocytes after 4 h (Fig. 2A) and a 2.5-fold increase in PCCL3 cells after 2 h (Fig. 2B). VEGF-A mRNA expression returned to control after 6 h of iodide deficiency in each case. Iodide deprivation increased VEGF-A protein expression (Fig. 2, C and D) after 2 (2.5-fold increase in human cells and 7.5-fold increase in PCCL3, P < 0.05) and 4 h (3.2-fold increase in human cells and 7.5-fold increase in PCCL3, P < 0.05). In control cultures, VEGF-A was released slowly and accumulated in culture medium. After iodide removal, VEGF concentration increased markedly, with a significant difference (P < 0.05) at 4 h, relative to controls, in human thyrocytes (Fig. 2E) and PCCL3 cells (data not shown).

VEGF-A was detected in the cytoplasm of human thyrocytes incubated with iodide. Of note, I was not detected in the cytoplasm of cells incubated without iodide for 4 h (Fig. 1). Hence, a rapid drop in intracellular I contents occurs in thyrocytes when they are incubated in a culture medium without iodide.

The acute iodide deprivation induced a 1.7-fold increase in VEGF-A mRNA expression (P < 0.05) in human thyrocytes after 4 h (Fig. 2A) and a 2.5-fold increase in PCCL3 cells after 2 h (Fig. 2B). VEGF-A mRNA expression returned to control after 6 h of iodide deficiency in each case. Iodide deprivation increased VEGF-A protein expression (Fig. 2, C and D) after 2 (2.5-fold increase in human cells and 7.5-fold increase in PCCL3, P < 0.05) and 4 h (3.2-fold increase in human cells and 7.5-fold increase in PCCL3, P < 0.05). In control cultures, VEGF-A was released slowly and accumulated in culture medium. After iodide removal, VEGF concentration increased markedly, with a significant difference (P < 0.05) at 4 h, relative to controls, in human thyrocytes (Fig. 2E) and PCCL3 cells (data not shown).

Fig. 4. Iodide deprivation increases HIF-1α activity. A: effects of iodide deprivation on HIF-1α protein activity in human thyrocytes were analyzed using an activity assay. Results are expressed as means ± SE of 5 independent wells (n = 5) of 1 representative experiment. Deferoxamine (DFO)-treated cells were used as positive control. ND, not detected. B: the involvement of HIF-1α as an inductor of VEGF-A gene expression was analyzed in cells treated with echinomycin (100 nmol/l). PCCL3 cells were iodide-deprived for 2 (ELISA) and/or 4 h (qRT-PCR, Western blot, ELISA). VEGF-A mRNA levels (B, left) were measured by qRT-PCR and normalized to β-actin. VEGF-A protein expression was analyzed by Western blot (B, middle), and densitometric values were normalized to β-actin. Values are expressed as means ± SE of 1 representative experiment; n = 5. *P < 0.05 vs. controls. VEGF-A release in the culture media was measured by ELISA (B, right). Results are expressed as means ± SE; n = 5. *P < 0.05 vs. 2-h-treated cells of the same group.

HIF-1α activity was measured in nuclear extracts. In cells incubated with iodide, HIF-1α activity was not detected, whereas in cells incubated without iodide, HIF-1α activity was induced after 1 h and sustained for ≤6 h of iodide deprivation (Fig. 4A). To firmly establish the link between HIF and VEGF, cells were incubated with echinomycin, a substance that inhibits the binding of HIF-1α to HRE located in the promoter region of the VEGF-A gene (Fig. 4B). Iodide deprivation-induced VEGF-A mRNA and protein expression, as well as release, were completely blocked after echinomycin treatment.

Increased ROS production in thyrocytes is the link between iodide deprivation and HIF-induced VEGF-A release. Bearing in mind that increased ROS production could be the link between iodide deprivation and activation of the HIF/VEGF pathway, we detected ROS by using DCFH-DA fluorescence. Little or no DCFH-DA fluorescence was detected in control cells (Fig. 5A). Conversely, DCFH-DA fluorescence was heavily enhanced after 1 and 2 h of iodide deprivation, consistent with a marked increase in ROS production. To test whether increased ROS production plays a role in iodide deprivation-induced HIF and VEGF production, PCCL3 cells (Fig. 5B) and human thyrocytes (Fig. 5C) were incubated with...
NAC. In control cells, NAC treatment influenced neither VEGF-A mRNA, protein expression, nor HIF-1α protein expression. Noteworthy was that increased VEGF-A mRNA and protein expression and HIF-1α protein expression induced by iodide deprivation were completely abolished by NAC. Blocking iodide transport induces the same effect as iodide deprivation. When human thyrocytes (Fig. 6A) or PCCL3 cells (Fig. 6B) were incubated with iodide and treated with perchlorate (1 mmol/l) for 4 h, an increase in VEGF-A mRNA and HIF-1α protein expression induced by iodide deprivation were completely abolished by NAC.

**DISCUSSION**

A role for increased secretion of VEGF in promoting angiogenesis in the thyroid during iodine deficiency in vivo has been described previously (13–15, 39, 41). To understand how thyrocytes transmit the angiogenic signal to adjacent endothelial cells, we have developed an in vitro model of iodine deficiency here. Our data demonstrate an increase in VEGF-A expression and secretion from thyrocytes immediately following abrupt iodide withdrawal (or when iodide uptake is blocked), suggesting that an acute drop in intracellular storages may trigger the early angiogenic phase observed during goiter formation (14). Changes in VEGF mRNA expression resulted from the sudden stop in iodide delivery to cells and not from the absence of iodide in culture media, because cells incubated without iodide from the beginning of the culture were still able to express VEGF mRNA at levels not statistically different (they were even slightly lower) from those of control cells incubated with iodide.

The increase in VEGF-A protein expression occurred shortly before the increase in mRNA expression, especially in human thyrocytes. Likewise, discrepancies were observed in the amplitude of mRNA and protein synthesis. Although we cannot provide a definitive explanation for these observations, it makes sense to propose that this is likely due to the specific regulation of VEGF-A. Hence, VEGF-A regulation has been reported to occur at different levels. In addition to the tran-
Fig. 6. Blocking iodide transport increases VEGF-A and HIF-1α expression. Human thyrocytes (A) and PCCL3 cells (B) were iodide-deprived for 4 h or treated with NaClO4 (1 mmol/l) in the presence of iodide for 4 h. A and B, left: VEGF-A mRNA was measured by qRT-PCR and normalized to β-actin. A and B, right: HIF-1α protein was analyzed by Western blot. Densitometric values were normalized to β-actin. Results are expressed as means ± SE of 5 independent wells (n = 5) of 1 representative experiment except for HIF-1α in human thyrocytes, where n = 2. *P < 0.05 vs. controls.

Fig. 7. Proposed mechanism of iodide deprivation-induced angiogenesis in thyroid angiofollicular units. In conditions of normal iodide supply (~150 μg/day), the trace element is actively transported into the cell through sodium iodide symporter (NIS), which is located at the basolateral plasma membrane. Iodide is then transported across the apical plasma membrane, where it is oxidized and bound into tyrosyl residues of thyroglobulin in a process that requires a peroxidase (TPO) and H2O2 provided from a NADPH oxidase (DUOX). As soon as iodide intracellular levels drop secondary to an acute decrease in iodide availability, an increase in intracellular ROS levels is observed. Increased oxidative load then contributes to stabilize HIF-1α, which forms a heterodimer along with HIF-1β (arrow b). This heterodimer binds to a hypoxia response element (HRE) site localized in the promoter region of the VEGF-A gene and turns on its expression (arrow a). As a result, VEGF-A protein is synthesized and released from thyrocytes. VEGF-A stimulates the proliferation of adjacent endothelial cells that belong to the same angiofollicular unit. This likely makes the total effective clearance rate of the thyroid rising as one of the several compensatory mechanisms against iodide scarcity. The source of increased ROS is not yet defined. ROS could be generated from DUOX (H2O2) or from mitochondria (mitoch) and/or endoplasmic reticulum (ER) as cause/consequence of the cellular stress that represents for thyrocytes the acute drop in iodide intracellular stores.
criptorial regulation, VEGF-A may for instance be regulated at translational and posttranslational levels (22, 42). Likewise, VEGF-A can be regulated at a translational level by mRNA capping proteins (16) or through the utilization of different ribosomal entry sites within the VEGF 5′-untranslated region (2, 5, 33). At a posttranslational level, VEGF-A protein may be glycosylated, thereby affecting VEGF-A secretion (22, 43). Further studies are needed to solve this question.  

HIF-1 has been identified as a main regulator of VEGF-A gene transcription, and a HRE exists in the promoter of the VEGF gene (1, 42). The synthesis of HIF can be activated within minutes when required (1, 3). Here we demonstrate a clear relationship between increased HIF-1α synthesis and VEGF-A production in response to an acute withdrawal of iodide in human thyroid cells. Moreover, the rapid time course of the increase in HIF-1α concentration and its specific inhibition by echinomycin [a specific inhibitor of the binding of HIF-1α to the HRE of the VEGF-A gene (21)] are consistent with a role for HIF-1α in the iodide-dependent regulation of VEGF-A production. Our finding that increased HIF-1α protein expression was not accompanied by an increased mRNA expression suggests a posttranscriptional mechanism of HIF-1α regulation. This is really not surprising since earlier findings showed that HIF-1α protein regulation occurs mainly via the stabilization of the protein rather than increased synthesis (1). Hence, HIF-1 is a heterodimer of two proteins, HIF-1α and HIF-1β. Whereas HIF-1β is constitutively expressed and not affected by oxygen, HIF-1α, which is also constitutively synthesized, is continuously degraded during normoxia. The von Hippel-Lindau tumor suppressor protein actually binds to the oxygen-dependent degradation domain located in the central region of HIF-1α, thereby leading to the degradation of HIF-1α through the ubiquitin-proteasome pathway. By contrast, this ongoing protein degradation is suppressed under hypoxic conditions (9, 18, 19, 24, 25, 28).

Overall, our results indicate that HIF is responsible for VEGF-A gene activation in thyrocytes following acute iodide deprivation (see Fig. 7, arrow a). Therefore, we propose that the acute drop in intracellular iodide levels may be perceived by thyrocytes as a “pseudohypoxic” stimulus that in turn triggers the HIF pathway. Previous papers reported that ROS produced from mitochondria or exogenous H$_2$O$_2$ may stabilize HIF-1α (3, 34, 38). Therefore, we propose that increased levels of ROS, as observed in condition of iodide deprivation (see Fig. 7, arrow b), may contribute to the stabilization of HIF-1α. This hypothesis likely makes sense since the induction of both VEGF-A and HIF-1α was completely abolished when ROS production was blocked by NAC. Although NAC may influence the basal thyroid cell function by decreasing the expression of NIS and TPO, NIS expression is unaffected, indicating that NAC treatment used for a short time period, as in the present experiments, does not alter the transport of iodide (31). The source of ROS eventually involved in this process remains unknown. It may perhaps be associated with H$_2$O$_2$, which is of utmost importance for thyroid hormone synthesis. ROS may also be generated from mitochondria or from endoplasmic reticulum as a cause or consequence of the “cellular stress” associated with acute iodide deprivation. Further investigations are ongoing to determine the source and the nature of ROS produced in such conditions and, more importantly, to find out the link between the drop in intracellular iodide and increased ROS levels. Despite these limitations, our data suggest that iodide deprivation-induced intracellular ROS production stabilizes the HIF-1α protein that in turn contributes to the expression and secretion of VEGF-A.

In conclusion, our results bring new insights into at least one mechanism underlying the early angiogenesis driven from iodide-deprived thyrocytes. The signal for the microvascular expansion that facilitates enhanced delivery of iodide appears to originate from the thyrocytes themselves and includes ROS production, stabilization of HIF-1α, and VEGF release (Fig. 5). This is the first description of such a chain of events linking reduced availability of a trace element, i.e., iodide and physiological angiogenesis. Because the iodide symporter NIS is expressed in organs other than the thyroid (trophoblast, breast, ovaries, salivary glands, stomach, cardiomyoblasts) (4, 8, 20, 26, 35, 36), it might be proposed that a similar mechanism for VEGF-A regulation by iodide exists in cell types other than thyrocytes. Whether this newly described regulatory pathway exists in other cell types or for other trace elements remains to be elucidated.

**GRANTS**

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