Acute iodine deficiency induces a transient VEGF dependent microvascular response in mammary glands involving HIF-1, ROS and mTOR

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Authors' contribution
J. Vanderstraeten is the main author; she designed and performed all the experiments in the framework of her PhD. She analyzed and interpreted the data and wrote the manuscript.

H. Derradji contributed to the analysis of the data and revised the draft of the manuscript.
P. Sonveaux contributed to the design of Laser Doppler measurements and revised the draft of the manuscript.

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Abstract

Iodine deficiency (ID), which affects almost two billion people worldwide, is associated with breast pathologies such as fibrosis in human and induces breast atypia in animal models. Because ID induces vascular activation in the thyroid, another iodide-uptaking organ, and as breast is also sensitive to ID, we aimed to characterize ID-induced effects on the breast microvasculature in vivo and in two different breast cell lines in vitro.

Virgin and lactating NMRI mice received an iodide-deficient diet and a Na\(^+\)/I\(^-\) symporter inhibitor for one to 20 days. Some virgin mice were treated with vascular endothelial growth factor A (VEGF) or VEGF receptors inhibitors. In vitro, ID was induced in MCF7 and MCF12A cells by replacing the iodide-containing medium by an iodide-deficient medium.

In vivo, VEGF expression was increased following ID in mammary tissues. Consequently, ID induced a transient increase in mammary gland blood flow, measured after anesthesia, in virgin and lactating mice, which was repressed by VEGF or VEGF receptor inhibitors. In MCF7 cells, ID induced a transient increase in reactive oxygen species (ROS), followed by an increase in hypoxia-inducible factor-1\(\alpha\) (HIF-1\(\alpha\)) protein and VEGF mRNA expression. Antioxidant N-acetylcysteine and mammalian target of rapamycin (mTOR) inhibitor blocked ID-induced HIF-1\(\alpha\) protein increase and VEGF transcription. However, mTOR activity was not inhibited by N-acetylcysteine. Similar responses were observed in MCF12A cells.

These data indicate that ID activates the canonical VEGF pathway and mTOR in breast tissues, which provides new insights to better understand the correlation between ID, vascular activation and breast pathologies.

Keywords:

Breast epithelial cells, mammalian target of rapamycin (mTOR), reactive oxygen species (ROS), vascular endothelial growth factor (VEGF), iodine deficiency.
Introduction

In mammals, iodine is an essential trace element required for several physiological purposes. Its supply must be ensured through adequate diet. However, a recent survey showed that still 25 countries out of 153 studied are considered iodine deficient (24). Beside the thyroid that processes iodide for hormone synthesis, the breast secretes iodide into the milk in order to meet the needs of the progeny and therefore increases iodide uptake at the end of pregnancy and during lactation through increased Na\(^+\)/I\(^-\) symporter (NIS) expression (6; 44). Because of increased needs in iodide, the population of pregnant or breast-feeding women suffering from iodine shortage is often higher compared to the normal population (43). While molecular iodine uptake by normal and cancerous virgin mammary gland is not dependent on NIS and seems to depend on facilitated diffusion in breast cancer cell line MCF7 (2; 16), iodide transport in non-lactating breast is debated due to low NIS expression (21; 32; 47; 55). However, iodine/iodide supply seems to be required for the maintenance of healthy mammary glands. Several studies have indeed highlighted changes induced by iodine deficiency (ID) in mammary glands and linked ID to mammary pathologies. For example, an acute drop in dietary iodide in rats caused atypia and dysplasia in the parenchyma as well as functional alteration in mammary glands (12). Similar alterations in breast morphology were seen in mice treated with NIS inhibitor perchlorate, although the implication of NIS in the transport of iodide in the mammary gland of non-lactating animals is still debated (13). Upon ID or perchlorate treatment, above mentioned changes in the breast were not abrogated when the animals were administered with prohormone thyroxin, suggesting that they were directly due to a lack of inorganic iodide and not to induced hypothyroidism (13).

Notably, perturbations in iodine/iodide supply have been linked to breast pathologies. Iodide was suggested to offer antioxidant protective effects in NIS expressing organs during reproductive and non-reproductive periods (49; 56) and several epidemiological and
molecular studies have attributed to iodine/iodide a protective role against breast pathologies. It was indeed shown that iodine and to a lesser extent iodide are able to reduce fibrocystic diseases (20) and to induce apoptosis and a reduction in breast tumor size (48; 50; 52). Several epidemiological studies have also shown a lower prevalence of breast tumors in diverse populations known for their high iodine/iodide intake (9; 36; 42; 45; 64) and it was proposed that high seaweed consumption, which is a source of high iodine/iodide supply, could have a protective role against breast cancer (4). Likewise, the prevalence of some breast pathologies is associated with some thyroid conditions known to be associated with ID such as enlarged thyroid (51). Moreover, the fact that iodine level in breast cancer tissues is lower than that in normal tissues also endorses a hypothetic protective role of iodine in the breast (30).

Because iodine/iodide seems to be important for mammary glands health, it is therefore worth investigating whether the breast has developed mechanisms to cope with iodine/iodide scarcity. Due to the primordial role of iodide in thyroid functions, thyroid pathologies associated with ID have largely been documented. The thyroid has different mechanisms to face ID that, in our opinion, are worth considering if relevant for the breast situation. One strategy consists in activating the microvasculature (19). We have previously shown in vitro and in vivo that, when facing short periods of acute ID, the level of reactive oxygen species (ROS) increases in thyrocytes, which increases local blood flow via a VEGF dependent pathway, independently of changes in thyroid-stimulating hormone production, probably in order to rapidly improve iodide supply (18; 19). As this effect does not depend on TSH but rather on the ability of thyrocytes to sense ID (18), we addressed the possibility that, similar to thyrocytes, mammary cells could activate the local microvascularization in response to iodide shortage and we report the existence of molecular pathways activated by acute ID in mammary glands.
Material and methods

Animal models

All *in vivo* experiments were conducted under approval of the Université catholique de Louvain (UCL) authorities (*Comité d’Ethique Facultaire pour l’Expérimentation Animale*) according to national and European animal care regulations (*European Directive 2010/63/EU and Royal Decree of the 29th of May 2013 relative to the protection of lab animals*) (authorization # 2014/UCL/MD/011 and 2017/UCL/MD/025). Mice were kept in the conventional animal facility where adequate room temperature and humidity for rodents were daily monitored and where a 12h/12h dark/light cycle was maintained. Mice well-being was examined every day.

To induce acute ID, wild-type outbred NMRI mice (strain 008, Harlan, Boxmeer, The Netherlands) were fed with a low-iodine diet (LID; 0.1 µg iodide/kg; Animalabo, Brussels, Belgium) supplemented or not with 1% sodium perchlorate in tap water for 1 to 20 days, or with a LID and tap water. Control animals received a normal diet (0.4 mg iodide/kg, AO3, Scientific Animal Food and Engineering, Augy, France) and tap water. Animals were randomly distributed among experimental groups, with 3 to 4 animals per cage. Virgin mice were eight or 12 week old and lactating mice 12 week old at the time of sacrifice; lactating mice were sacrificed in the third week of lactation and pups were separated from their mother 30 minutes before the blood flow measurement.

For inhibition of the VEGF pathway, wild-type NMRI mice (Harlan, Boxmeer, The Netherlands) were either fed with a normal diet (controls) or with a low-iodine diet supplemented with 1% sodium perchlorate in drinking water for 2 days. At the beginning of the ID treatment, half of the groups were injected intraperitoneally with a saline solution of bevacizumab (10 mg/kg; Roche, Welwyn Garden City, UK) or with a solution of SU5416 (25 mg/kg; VWR, Pennsylvania, United States) in dimethyl sulfoxide (DMSO) to inhibit VEGF-
A and VEGF receptors 1 and 2, respectively. Control animals were injected with an equimolar solution of irrelevant human IgGs (Sigma, St.-Louis, MO, USA) as a control for bevacizumab or with DMSO as a control for SU5416 treatment. To inhibit mTOR, 4 mg/kg/day of rapamycin (VWR) in a saline solution with 4% ethanol, 0.2% sodium carboxymethylcellulose (Sigma) and 0.25% Tween 80 (Sigma) or vehicle solution only was injected intraperitoneally to mice. Mice were never exposed to ID, to the inhibitor or any other medicine prior to the treatment.

**Blood perfusion measurements and tissue sampling**

Mice were anesthetized with 85 mg/kg Ketamine (Anesketin, Eurovet, Belgium) and 10 mg/kg Xylasine (Rompun, Bayer HealthCare, Belgium) and placed on a heating pad (35°C). A maximum of 12 mice was analyzed per day, with two or three mice per experimental group on the same day. Mice blood perfusion was measured at the same hour that the treatment was started one to 30 days before the measurement. The area around the second right mammary gland starting from the tail was shaved. Its blood flow was measured using a Laser Doppler imager (Moor Instruments, Axminster, UK). Glands were identified and delineated based on anatomical images, and blood flow was quantified in the areas of interest from colored histogram pixels. After measurements, mice were overdosed with Pentothal (Abbott, Louvain, Belgium). Mammary glands were excised, fixed in 4% of paraformaldehyde in PBS for 36 h, and embedded in paraffin. Thick sections (5 µm) were used for immunohistochemistry and immunofluorescence. A portion of mammary tissue of lactating mice was frozen in liquid nitrogen and used for RNA and protein analyses.

**Cell models**

MCF7 human mammary gland adenocarcinoma cells (ATCC, Manassas, USA) were routinely cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley,
UK) supplemented with 10% of fetal bovine serum, 100 U/ml (50 µg/ml) penicillin-
streptomycin (Invitrogen) and 2.5 µg/ml fungizone (Invitrogen). MCF12A human normal
mammary gland cells (ATCC) were cultured in a 1:1 mixture of DMEM and Ham’s F12
medium, supplemented with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera
toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, 5% horse serum, 100 U/ml (50
µg/ml) penicillin-streptomycin and 2.5 µg/ml fungizone. HDF human dermal fibroblasts
(PromoCell, Heidelberg, Germany) were cultured in DMEM (Invitrogen) supplemented with
2% fetal calf serum (Invitrogen), 1% L-glutamine (Sigma), 100 U/ml (50 µg/ml) penicillin-
streptomycin and 2.5 µg/ml fungizone. Cells were cultured at 5% CO₂, 37°C in a humidified
atmosphere, as previously described (18). Seven days before the experiments, 10⁻⁸ M NaI was
added to the medium. For the experiment with trans-retinoic acid (tRA), 1µM tRA (Sigma)
was added 24 h before acute ID induction. On the day of the experiment, 0.75 mM of
antioxidant N-acetylcysteine (NAC, Sigma) was added to the medium for 1 h before medium
change where indicated. After washing with PBS, culture medium was replaced by fresh
medium containing NaI (controls) or not (ID) with or without 10 nM of mTOR inhibitor
rapamycin (LC Laboratories, Woburn, USA), 0.75mM of NAC or 100 nM of echinomycin
(Alexis Biochemicals, California, USA) where indicated. Cells were harvested at different
time points after medium renewal.

Western blotting

Cell and tissue samples were homogenized in Laemmli buffer (10% glycerol, 2% SDS, 50
mM Tris-hydroxymethylaminomethane, 5 mM EDTA in 100 ml distilled water) containing a
protease inhibitor cocktail (Roche, Mannheim, Germany), and sonicated for 15 s. Protein
content was determined using a BCA protein assay kit (Pierce, Rockford, IL). Western blots
were performed as previously described (Gérard 2012). Primary antibodies were incubated
overnight at 4°C and secondary antibodies for 1 h at room temperature (Table 1). Staining was visualized using the Supersignal West Pico/Femto chemiluminescence kit of Thermo Scientific (Waltham, MA). Exposure times are given in table 1. Protein expression was quantified by densitometry using NIH Scion Image Analysis Software (National Institutes of Health, Bethesda, MA). Data were normalized to β-actin.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (17). Antigen retrieval was necessary for VEGF-A and CD31 detection. To that aim, tissue sections were microwaved in citrate buffer (0.01 M, pH 6.0) for one cycle of 3 min at 750 W, followed by 4 cycles of 3.5 min each at 350 W. Nonspecific staining was prevented by incubating sections with non-immune goat serum (1:50; Vector Laboratories, Burlingame, CA) in PBS/BSA 5% for 30 min at room temperature. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 h at room temperature (Table 2). Primary antibodies were omitted in negative controls. Peroxidase activity was revealed using 3,3′-diaminobenzidine (Dako). Sections were counterstained with Mayer’s hematoxylin. Tissues were imaged on an Axio scope A.1 microscope (Zeiss) equipped with a DS-5Mc color digital camera head (Nikon, Amsterdam, The Netherlands). Anti-VEGF antibody specificity was assessed as described before (19). Stained surface was quantified with a brightfield immunohistochemistry image analysis program (Leica Biosystems, Wetzlar, Germany).

For staining quantification, sections were digitalized at a 20x magnification using an SCN400 slide scanner (Leica, Wetzlar, Germany). The tissue in each section was delineated manually and care was taken to exclude tissue folds, bubbles and artefacts from the analysis. Scanned slides were then quantified using Tissue IA (Leica Biosystems, Dublin, Ireland). Color deconvolution was applied to each pixel using hematoxylin and DAB matrices of the
software. On the DAB matrice, a threshold was adjusted for DAB detection according to intensity (grey values from 0 to 255) on representative stained versus not stained tissue areas. In a similar way, a threshold was also adjusted for tissue detection. These parameters were kept constant throughout the study for each immunostaining. Results were expressed as stained area /tissue area.

Quantitative polymerase chain reaction

For quantitative polymerase chain reaction (RT-qPCR), cell and tissue samples were homogenized in 1 ml TriPure isolation reagent (Roche), and total RNA was extracted according to the manufacturer’s protocol and resuspended in RNase-free water. Reverse transcription was performed as previously described (18). cDNAs (0.04 µg of cDNA template in 2 µL) were admixed with 10 µM of each selected primer pair (Table 3) and Perfecta SYBR green reaction mix (VWR) in a final volume of 15 µL. Reactions were performed with an IQ5 iCycler (Bio-Rad, Herts, UK) as previously described (18). Annealing temperatures are given in Table 3. Amplification levels were normalized to those of β-actin. All samples were measured in duplicate.

ROS measurements

ROS production was measured using fluorescent dye Carboxy-DCFDA (5-(and-6)-Carboxy-2',7'-Dichlorofluorescein Diacetate) (DCFDA, Invitrogen). Cells were plated in multichamber glass slides (Sigma) in medium containing 10^{-8} M of NaI until they reached 80% confluence. After washing with PBS, medium was replaced by fresh medium containing NaI or not. After 2, 4 or 6 h, cells were washed with PBS and incubated in a Krebs-Ringer HEPES (pH 7.4) solution without albumin and containing DCFDA (25 mM) for 1 h at 37°C, 5% CO₂. Slides were washed 3X3 min with PBS, and nuclei were stained with Hoechst dye (ThermoFisher;
Excess dye was removed with PBS, and slides were mounted in fluorescent mounting medium (Dako, Heverlee, Belgium). ROS production was visualized on a fluorescence microscope (Zeiss, Axio scope A.1, Zaventem, Belgium).

**Immunofluorescence**

Cells were plated in multichamber glass slides (Sigma) in medium containing 10^{-8} M of NaI until they reached 80% confluence. After washing with PBS, medium was replaced by fresh medium containing NaI or not. After 4 h, cells were washed with PBS and fixed with paraformaldehyde 4% for 10 minutes. Cells were washed and then quenched with NH4Cl 50mM in water (sodium borohydride, Sigma-Aldrich) for 5 minutes, twice. After washing, cells were blocked for 1 h in PBS/BSA 5%/saponin 0.1%, and incubated at 4°C overnight with primary antibodies (4-HNE, 393207-100, VWR, 1/500; previous use of the antibody: (38)) in PBS/BSA 1%/saponin 0.1%. Primary antibodies were omitted in negative controls. Signals were revealed after 1h incubation at room temperature in the dark with species-specific secondary antibodies conjugated to fluorophores (Alexa Fluor 488-conjugated goat anti-rabbit, A11034, Invitrogen, 1/300) in PBS/BSA 1%/saponin 0.1%. Nuclei were stained for 5 minutes with 4′-6′diamidino-2-phenylindole (DAPI, 1/10,000) and slides were mounted in Fluorescent Mounting Medium (Dako Cytomation). Images were captured on an AxioCam MRC5 fluorescence microscope using the Axio Vision 4.8 software (Zeiss).

**Statistics**

All data are expressed as means ± SEM. *In vivo* experiments were independently repeated 2 or 3 times with 3 animals in each group. *In vitro* experiments were independently conducted 3 to 11 times with 3 to 6 replicates for each condition. Unpaired Student's *t* test and one-way
ANOVA with Dunnett, Tukey-Kramer or Bonferroni post-hoc test were used where indicated (GraphPad Instat, San Diego, CA). P < 0.05 was considered to be statistically significant.
Results

Acute ID induces a transient increase in blood flow and VEGF expression in lactating mammary glands

Iodide uptake and NIS expression by the mammary gland are strongly enhanced during lactation (6). Therefore, we initially studied the effects of acute ID in lactating mice. We first investigated the impact of ID on mammary gland blood flow and microvasculature. Compared to control, ID increased blood flow from day 1 to day 10 of the treatment (Fig. 1a). This increase was maximal after 10 days and then decreased to reach back control level after 15 days of treatment. To rule out the potential toxic or off-target effects of perchlorate in this response, a group of mice received LID food without perchlorate for 4 days, which also induced a significant increase in blood flow (Fig. 1b).

VEGF has been involved in the response of the thyroid vasculature to ID (19). In the mammary gland, RT-qPCR analyses detected a significant increase in VEGF mRNA expression at day 1 of ID (Fig. 1c). It went along with increased VEGF protein expression detected by immunohistochemistry around some blood vessels and in the stroma (Fig. 1d-g). This staining was strongly enhanced from day one (Fig. 1e-f) to day 10 of the treatment. Increased staining was also observed in the lumen of the glands and at the apical membrane of the mammary epithelial cells (Fig. 1g). To assess the involvement of VEGF in the ID-induced increase in blood perfusion, lactating mice were injected with a VEGFR2/1 inhibitor, SU5416, or with the vehicle solution, DMSO. SU5416 prevented the ID-induced increase in blood perfusion, confirming VEGF role this response (Fig. 1h).
Acute ID induces a transient VEGF-dependent increase in blood flow in virgin mammary glands

Although iodide uptake is very low in non-lactating mammary glands compared to lactating glands (6), acute ID also induced an increase in blood flow in the mammary glands of 8-week old virgin mice (Fig. 2a). Increased perfusion was detected only at day 1 of treatment. Blood flow then progressively declined to basal level from day 2 to day 10 (Fig. 2a). A second experiment with longer treatment times revealed no increase in blood flow after ID exposure up to 20 days (Fig. 2b). Mice that received LID food without perchlorate for 2 days also experienced increased mammary gland blood flow (Fig. 2c). To verify that the difference in age between lactating and virgin mice was not responsible for the difference in the response induced by ID, 12-week old virgin mice were exposed to ID for 1, 2, 4 or 10 days and their blood perfusion was measured. A similar response was observed (data not shown).

To investigate the effects of acute ID on vascularization, blood vessels were labelled with an anti-CD31 antibody. An increase in total stained surface was observed at day 2, showing that the total vascular surface was increased upon ID (Fig. 2d). However, ID did not induce an increase in the number of vessel sections per unit of surface (Fig. 2e), indicating that vasodilation, not angiogenesis, could be responsible for increased blood flow in the mammary gland upon acute ID. This was associated to changes in VEGF expression. In control mice, weak VEGF staining, studied by immunohistochemistry, was observed in few mammary epithelial and endothelial cells and in the stroma (Fig. 2f-g). Comparatively, VEGF expression was enhanced on days 1 and 2 of ID at all 3 locations (Fig. 2h-i). After 4 days of ID, VEGF expression decreased but was still higher than in controls. At day 10, VEGF expression was similar to the expression in controls.

To determine whether VEGF is responsible for ID-induced increase in blood flow, mice were injected either with VEGFR2/1 inhibitor SU5416 or with DMSO as a control.
SU5416 inhibited the increased blood flow in response to acute ID, whereas basal blood flow in iodine-replenished mice was not affected (Fig. 2j). Similar data were obtained when using VEGF-blocking antibody bevacizumab at a high dose instead of SU5416 (Fig. 2k), thus indicating that VEGF is at least involved in the increased mammary gland perfusion in iodine-deprived virgin mice.

Acute ID induces a ROS and mTOR-dependent increase in HIF-1α protein and VEGF mRNA expression in MCF7 cells

VEGF expression was increased during acute ID in both lactating and virgin glands. We therefore aimed to elucidate the underlying molecular pathway. To do so, we used two NIS expressing cell lines: MCF7, a breast cancer cell line, and MCF12A, a non-cancerous cell line. NIS expression was compared in MCF12A and in MCF7 cell lines in control experiments. NIS mRNA expression was considerably higher in MCF7 cells than in MCF12A cells while NIS protein expression was not significantly different in both cell lines (Fig. 3a, b).

The effects of acute ID on VEGF mRNA expression in MCF7 cells were studied after 2, 4, 6, 8 and 24 h showing that acute ID induced a transient increase in VEGF mRNA expression only at 6 h (data not shown). Thus, we used this timing (Fig. 4a) for the following experiments. Iodide uptake and NIS expression were shown to increase with trans-retinoic acid (tRA) treatment (32). Cells were thus treated with tRA to see whether NIS and iodide uptake induction could influence the response to ID. However, though the addition of tRA increased NIS and VEGF mRNA expression, it did not influence the amplitude of the increase in VEGF mRNA induced by acute ID (data not shown). Because iodide is an antioxidant (57) and ROS are able to influence VEGF expression (37; 63), we analyzed ROS content and found that iodine-deprived cells had increased ROS levels 4 h after ID (Fig. 4b-e). Oxidative
damage was then assessed through 4-HNE adducts measurement by immunofluorescence. An increase in 4-HNE staining was observed at 4 h of ID (Fig. 4f-g). A causative link between increased ROS and VEGF mRNA expression in the context of acute ID was established by showing that antioxidant NAC prevented the increase in VEGF mRNA levels in cells exposed to ID (Fig. 4h). NAC had no effect on basal VEGF mRNA expression in control cells. Further, VEGF is a HIF-1-target gene (15), and transcription factor HIF-1 is activated by ROS that increase the expression of its α subunit (23). In line with an involvement of HIF-1 in the response to ID, we observed a significant increase in HIF-1α expression 4 h after ID (Fig. 4i). NAC prevented ID-induced HIF-1α protein overexpression (Fig. 4j), which revealed that HIF-1 can act downstream of ROS in the response of breast cancer cells to ID. Of note, NAC did not influence basal HIF-1α protein expression in iodide-replenished cells.

The activity of the transcription factor HIF-1 was then inhibited by echinomycin. As a consequence, the ID-induced effect on VEGF mRNA overexpression was not observed (Fig. 4k).

In thyrocytes, mTOR promotes HIF-1α and VEGF expression during ID (7), and mTOR activity can be increased by ROS (5). We therefore reasoned that mTOR could be involved in the response of breast cancer cells to ID. Accordingly, phosphorylation of P70S6K on threonine 389, an established target of mTOR (46), significantly increased in MCF7 cells following acute ID (Fig. 5a). P70S6K phosphorylation was increased as soon as 2 hours after ID induction (fig.4d), indicating that mTOR activity is enhanced before the HIF-1α rise. This response was not reduced by NAC (Fig. 5b) which suggests that mTOR activation by ID is not dependent on ROS production. Downstream, the mTOR inhibitor rapamycin inhibited ID-induced HIF-1α protein (Fig. 5c) and VEGF mRNA (Fig. 5d) expression, thereby showing the involvement of mTOR in ID-induced pathway that leads to VEGF overexpression. Rapamycin did not modify the basal levels of the HIF-1α protein and...
VEGF mRNA in iodine-replenished MCF7 cells (Fig. 5c-d). However, P70S6K phosphorylation in MCF7 cells was drastically reduced in the presence of rapamycin, indicating that the concentration used was efficient (data not shown).

Altogether, our results strongly suggest that acute ID induces a ROS and mTOR-dependent rise in HIF-1α protein that promotes VEGF mRNA overexpression.

Acute ID induces a ROS and mTOR-dependent increase in VEGF mRNA expression

MCF12A cells

We next verified whether acute ID could induce a rise in VEGF mRNA in another NIS-expressing breast cell line. Intracellular ROS content of MCF12A cells was increased after 2 h of ID, compared to controls (Fig. 6a-d) and 4-HNE staining was also more pronounced after 3 h of ID (Fig. 6e-f). It was followed by an increase in VEGF mRNA expression that was significant only at 3 h (Fig. 5g), but not at 2, 4, 6 or 24 h (data not shown). The increase in VEGF mRNA expression was repressed by NAC (Fig. 6h) and rapamycin (Fig. 6i). Similar to what was seen in MCF7 cells, neither NAC nor rapamycin influenced basal VEGF mRNA expression in MCF12A cells supplied with iodine (Fig. 6h-i). Our data suggest that the same molecular pathways are activated in MCF12A and in MCF7 cells during acute ID.

In order to assess whether ID-induced effects are specific to cells capable of iodide uptake, a control experiment was carried out with human dermal fibroblast (HDF) cells which do not express NIS. HDF cells exposed to ID for 4 or 6 h did not increase VEGF mRNA expression (data not shown).

Acute ID-induced blood perfusion increase in virgin mice is dependent on mTOR

Because our in vitro data unraveled mTOR contribution to ID-induced VEGF mRNA expression, virgin mice were exposed to rapamycin in order to verify that mTOR is also
involved in vivo. No increase in blood perfusion was induced by ID treatment in mice exposed to rapamycin, confirming mTOR involvement in ID-induced blood perfusion increase (Fig. 7).

Acute ID increases NIS expression in vitro and in vivo

It is likely that the ID-induced increase in blood perfusion is aimed at improving iodide supply, as previously hypothesized in the case of the thyroid gland (18; 19). However, mammary cells could further exploit other mechanisms, such as increasing NIS expression. It was therefore investigated in mammary cells in vivo and in vitro. In vivo, in lactating mammary glands, NIS mRNA expression, studied by qPCR, appeared to be non-significantly increased at the first day of ID treatment, and gradually decreased back to control level from day 4 to day 15 (Fig. 7a). NIS protein expression, studied by western blot, was significantly enhanced at day 10 of ID treatment (Fig. 7b). In vitro, in MCF7 cells, NIS mRNA expression increased after 4 hours of ID (Fig. 7c) and its protein expression transiently increased after 4h of ID (Fig. 7d). Nevertheless, in MCF12A cells, ID did not induce an increase in NIS mRNA and protein expression at the studied time points (Fig. 7e, f).
Discussion

Several studies have reported functional and histological changes in the breast tissues induced by ID and have suggested links between iodine supply and breast health (12; 13; 51; 54); but, unlike the molecular pathways activated by iodide/iodine supplementation that are well characterized (41; 50; 52), little is known about the pathways activated by an acute drop in iodide supply. Here, we report that acute ID activates a ROS/mTOR-HIF-1-VEGF pathway in the mammary cells, which induces vasodilation in the mammary gland.

We first observed that transient microvascular activation occurred when iodide intake dropped in virgin and lactating mouse mammary glands, i.e., two models with different iodide requirements (6). ID significantly increased VEGF expression and local perfusion in the two models, which was shown to be dependent on VEGF. This finding does not rule out the possible involvement of other factors. The VEGF-dependent increase in blood perfusion was independent of angiogenesis and could thus be explained by the vasodilatory activity of VEGF (22; 33). Indeed, VEGF binding to its receptors on endothelial cell membrane induces a cascade of events leading to endothelial nitric oxide (NO) synthase activation and/or up-regulation and a subsequent production of NO, a potent vasodilator (33; 60). In addition, VEGF can also induce endothelial cell proliferation without inducing the formation of new blood vessels but, rather, increasing their diameter, as was previously observed in thyroid (19). The involvement of NO and/or endothelial cell proliferation in our model needs further investigation.

Several factors triggering VEGF expression and microvascular activation were assessed in vitro in two different NIS-expressing cell lines: MCF7 breast cancer cells and MCF12A breast non-cancer cells. In both models, ID increased VEGF mRNA expression, which is in good agreement with the VEGF-dependent vascular activation detected in vivo.
Increased VEGF mRNA expression in the two cell lines in the absence of thyroid hormones and perchlorate indicate that breast cells are directly sensitive to ID alone and that the vascular activation observed in mice is not due to perchlorate itself or to changes in thyroid hormone levels. Furthermore, the increase in blood flow \textit{in vivo} occurred 1 day after ID, before any changes in thyroid hormone levels (19). Moreover, there were no more changes in the blood flow in hypothyroid condition after 15, 20 or 30 days. Likewise, a control experiment where mice only received a low iodide diet without perchlorate left out the possibility that vascular activation is an off-target effect of perchlorate.

In MCF7 cells, the expression of both VEGF mRNA and the \( \alpha \) subunit of transcription factor HIF-1 was induced by acute ID. In this cell line, ROS, mTOR and HIF-1 were required to induce VEGF transcription, as the inhibition of ROS and mTOR prevented both HIF-1\( \alpha \) and VEGF overexpression and the inhibition of HIF-1 prevented VEGF overexpression. The contribution of mTOR was further demonstrated in ID-induced blood perfusion increase \textit{in vivo}. Of note, rapamycin did not modify the basal levels of VEGF mRNA and HIF-1\( \alpha \) protein. This observation seems contradictory with the important role of mTOR in gene expression regulation, including HIF-1\( \alpha \). However, other groups obtained similar results in non-cancerous tissues (1) and in different cancer cell lines (31). In cancer cells such as MCF7 (58), HIF-1\( \alpha \) protein level is elevated under normoxic conditions; it is possible that mTOR plays a role in this overexpression, but other pathways could also be involved as well, such as Ras/Raf/MEK/ERK (26; 35). HIF-1\( \alpha \) can also be regulated by other factors that are not targeted by rapamycin, such as Hsp90 (25).

mTOR activation was not dependent on ROS. It is thus possible that two different pathways leading to VEGF overexpression are activated by acute ID. Thus, we propose that acute ID triggered ROS production, activated mTOR, and that both ROS and mTOR increased HIF-1\( \alpha \) expression that triggered VEGF transcription in these cells. We do not
exclude the possibility that other transcription factors are also involved in the pathway. Although the initial aim of our study was not to focus on ROS, a likely explanation for increased ROS content upon acute ID could be related to unrestricted production of ROS associated with the cellular respiration (65). A drop in the level of available iodide might reduce antioxidant mammary cell defenses against their own metabolic production of ROS. Other possible mechanisms are of course not excluded, such as the activation of ROS-generating NADPH oxidases by ID, for instance.

We currently do not know how mTOR is activated by acute ID. Several models have indeed shown that ROS could activate mTOR signaling (27), and we were surprised to observe that NAC did not prevent P70S6K phosphorylation by ID. Different hypotheses could be put forward as possible explanations for mTOR activation, leading to different experimental perspectives. ID might activate the PI3K/AKT signaling pathway, which is known to regulate mTOR. PTEN is a negative inhibitor of the AKT/mTOR pathway, of which the activity can be regulated by its redox state (34). Because iodide itself is an antioxidant (59), we can hypothesize that it could directly interact with PTEN and decrease the oxidized form of PTEN, which then leads to PTEN activation. A sudden drop in iodide availability could reverse this inhibition (474). Moreover, a role for the unfolded protein response (UPR) pathway could also be considered, as it is known to activate the mTOR pathway (29; 62). ID was shown to induce intracellular Ca^{2+} leaks via the RYRs in the thyroid (8), and such a mechanism is conceivable in breast cells, thereby potentially activating the UPR. It is also possible that the presence/absence of iodide influences disulfide bonds or affect the redox state of endoplasmic reticulum membrane lipids, and its sudden removal could therefore trigger the UPR (39; 61). Besides, I2 addition leads to iodolactone formation, which was shown to activate PARP-1 in MCF7 cells, among others (3). PARP-1 in turn can activate AMPK, as it was demonstrated in other models, which is a negative regulator of mTOR (14).
As mammary glands, and more specifically MCF7 cells, express the lactoperoxidase (53), it is possible that part of the iodide leads to I2 production, and a decrease in iodide could maybe lead to a decrease in iodolactones and thereby to mTOR activation.

While VEGF induction upon acute ID was observed in virgin and lactating mice as well as in two cell line models, the kinetics of its expression varied in the different models. In mice, blood flow induction, though transient in both models, lasted longer in lactating breasts.

This might reflect the increased requirement of iodide in breast during lactation, both for the mother and the progeny (43). However, it is also possible that estrogens, which are known to be able to stimulate angiogenesis (28; 40), might reinforce the effects of ID. Mammary glands could therefore react to ID through microvascular activation, the extent of which depending on the physiological state. In our assays, microvascular activation was accompanied by an increased expression of NIS, which was detected in lactating mice and in MCF7 cells, but not in MCF12A cells. This does not exclude a possible regulation of NIS in these cells after longer exposure time. Taken together, these data suggest that mammary cells are sensitive to ID and attempt to increase their iodide intake by increasing their surrounding blood flow and, in MCF7 cells and lactating mice, their transport activity. Of note, this reaction occurred only when cells faced a sudden decrease in iodide supply, as VEGF mRNA expression did not change in cells constantly exposed to ID and in cells constantly exposed to sodium iodide (data not shown).

In MCF12A cells, the same pathways are activated by acute ID as in MCF7 cells, though basal iodide uptake is low in both cell lines (32). VEGF transcription was induced by acute ID and it was also dependent on both ROS and mTOR. In MCF7 cells, the amplitude of ID-induced VEGF mRNA overexpression was not influenced by tRA, which is known to increase NIS expression and iodide uptake in those cells (32). Furthermore, virgin mice, which are known to have a very low iodide intake (6), also reacted to ID by increasing
mammary gland blood flow and VEGF expression. Conversely, a control experiment suggests that acute ID in fibroblasts, which are not known to require iodide, did not induce a rise in VEGF mRNA expression. Thus, this effect seems to be specific to cells that physiologically require iodide. The initial amount of intracellular iodide does not seem to affect the trigger of the reaction, but might influence the regulation of the reaction since it lasted longer in lactating females than in virgin females.
Conclusion

In conclusion, our results show that acute ID induces a VEGF-dependent microvascular activation in mammary glands. ID-induced HIF-1/VEGF pathway is dependent on mTOR signaling and on increased ROS content. This local vascular activation leading to a vasodilation and an increased blood flow could be viewed as a rapid response to ID that aims at improving iodide bioavailability to the breast tissue when facing iodide shortage, similar to what is described in the thyroid (18).
List of abbreviations

Hypoxia-inducible factor-1α (HIF-1α), iodine deficiency (ID), low iodide diet (LID), mammalian target of rapamycin (mTOR), N-acetylcysteine (NAC), quantitative polymerase chain reaction (qPCR), reactive oxygen species (ROS), sodium-iodide symporter (NIS), trans-retinoic acid (tRA), vascular endothelial growth factor A (VEGF).
Acknowledgements

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Competing interests

The authors declare that they have no conflict of interest.


Legends of the Figures

Figure 1. Acute iodine deficiency triggers perfusion and VEGF expression in lactating mammary glands in mice. Lactating NMRI mice received an iodine containing diet (control) or iodine-deficient diet with 1% sodium perchlorate (ID) in the drinking water for 1 to 15 days (d). (A-B) Blood flow in mammary glands was measured with a Laser Doppler flowmeter (A: control: N=10, ID 1 and 15 days: N=8, ID 4 and 10 days: N=9; B: N=3). (C) VEGF mRNA expression was determined using RT-qPCR on tissue samples (control and ID 15 days: N=8, ID 1, 4 and 10 days: N=7). (D-G) VEGF protein was detected using immunohistochemistry on mammary glands collected 1 day after ID or control treatment. Representative pictures of N = 8 are shown for control mice (D-E) and iodine-deprived mice (F-G). Scale bars = 50 µm. (H) Lactating mice exposed to ID or control treatment were injected with either DMSO or SU5416 diluted in DMSO (control N=4, ID 2 days, SU5416 and SU5416 ID 2 days: N=5). Data are expressed as means ± SEM. Statistical test: one-way ANOVA with Dunnett post-hoc test (A, C), Tukey-Kramer (H) or unpaired Student’s t test (B). * P < 0.05, ** P < 0.01, *** P < 0.001 versus control (A-C, H).

Figure 2. Acute iodine deficiency triggers VEGF expression that induces perfusion in mammary glands of virgin mice. Virgin mice received an iodine containing diet (control) or iodine-deficient diet with 1% sodium perchlorate (ID) in the drinking water or without perchlorate (LID) for 1 to 10 days or from 15 to 20 days (d). (A-C) Blood flow in mammary glands was measured with a Laser Doppler flowmeter (A: control: N=9, ID 1, 2, 4 and 10 days: N=8; B: control: N = 7, ID 15 and 20 days: N=6; C: N=3). (D-E) CD31 immunostaining on mammary glands collected 1 to 10 days after ID or control treatment was quantified from scanned slides (D) and sections into vessels were counted (E): control and ID 10 days: N=5, ID 1 day: N=7, ID 2 and 4 days: N=6; E: control: N=6, ID 1, 2 and 4 days: N=7, ID 10 days: N=5). (F-I) VEGF protein was detected using immunohistochemistry on mammary glands collected 1 day after ID or control treatment. Representative pictures of N = 8 are shown for control mice (F-G) and iodine-deprived mice (H-I). Scale bars = 20 (G, I) or 50 µm (F, H). (J) Virgin mice exposed to ID or control treatment were injected with either DMSO or SU5416 diluted in DMSO (control and ID 2 days: N=6, SU5416 and SU5416 ID 2 days: N=5). (K) Virgin mice exposed to ID or control treatment were injected with either IgG or bevacizumab (control IgG and control bevacizumab: N=6, ID 2 days IgG: N=5, ID 2 days, bevacizumab: N=7). (J-K) Blood flow in mammary glands was measured with a Laser Doppler flowmeter. Data are expressed as means ± SEM. Statistical test: one-way ANOVA with Dunnett post-hoc test (A, B, D, E), Tukey-Kramer (J, K) or unpaired Student’s t test (C). * P < 0.05, ** P < 0.01 (A-E, J-K).

Figure 3. NIS mRNA expression is higher in breast cancer cells than in non-cancer cells. MCF12A and MCF7 cells were cultured in iodine-containing medium for 7 days. (A) NIS mRNA expression was determined using RT-qPCR (N=2, one representative experiment). (B) NIS protein expression was determined by western blotting (N=2, one representative experiment). Data are expressed as means ± SEM. Statistical test: Unpaired Student’s t test. ** P < 0.01.
Figure 4. Acute iodine deficiency induces a ROS/HIF-1α-dependent increase in VEGF mRNA expression in MCF7 breast cells. MCF7 cells were cultured in iodine-containing medium for 7 days. Medium was replaced by iodine-containing (control) or iodine deficient (ID) medium with or without the antioxidant NAC. Cells were harvested 2, 4 or 6 hours (h) after medium change. (A) VEGF mRNA expression was determined using RT-qPCR (N=11). (B-E) ROS content was visualized with DCFDA and nuclei were stained with Hoechst fluorescent dye in control (B-C) and iodine deficient (D-E) cells (N=3). (F-G) 4-HNE adducts were detected using immunofluorescence after 4 h of ID (G) or control (F) treatment (N = 3). Representative pictures are shown. Scale bars = 20 µm. (H) The involvement of ROS in ID-induced VEGF mRNA expression was studied by western blotting thanks to the use of NAC (N=4). (I) HIF-1α protein expression was studied by western blotting (N=4). (J) The involvement of ROS in ID-induced HIF-1α protein expression was studied by western blotting thanks to the use of NAC (N=5). (K) The involvement of HIF-1α in ID-induced VEGF mRNA expression was studied by RT-qPCR thanks to the use of echinomycin (N=3). Data are expressed as means ± SEM. Statistical test: Unpaired Student’s t test (A) or one-way ANOVA with Dunnett (I) or Tukey-Kramer (H, J, K) post-hoc test. */+ P < 0.05, */++ P < 0.01, ***/+++P<0.001 *versus control; + versus ID 4/6h (A, H-K).

Figure 5. Increase in VEGF mRNA and HIF-1α protein expression depends on mTOR in MCF7 breast cells. MCF7 cells were cultured in iodine-containing medium for 7 days. Medium was replaced by iodine-containing (control) or iodine deficient (ID) medium with or without the inhibitor of mTOR, rapamycin. Cells were harvested 2 or 4 hours (h) after medium change. (A) P70S6K phosphorylation on threonine 389 was determined by western blotting (N=5). (B) The involvement of ROS in ID-induced P70S6K phosphorylation on threonine 389 was studied through the use of rapamycin (N=3). (C) The involvement of mTOR in ID-induced HIF-1α protein expression was studied by western blotting thanks to the use of rapamycin (N=5). (D) The involvement of mTOR in ID-induced VEGF mRNA expression was studied by RT-qPCR thanks to the use of rapamycin (N=4). Data are expressed as means ± SEM. Statistical test: one-way ANOVA with Dunnett (A), Bonferroni (B: comparing “control” to “ID 4h” and “NAC” to “NAC, ID 4h”) or Tukey-Kramer (C, D) post-hoc test. * P < 0.05, **/+ P < 0.01, +++ P<0.001. *versus control, +versus ID 4h.

Figure 6. Acute iodine deficiency induces a ROS/ mTOR-dependent increase in VEGF mRNA expression in MCF12A breast cells. MCF12A cells were cultured in iodine-containing medium for 7 days. Medium was replaced by iodine-containing (control) or iodine deficient (ID) medium with or without the antioxidant NAC or the inhibitor of mTOR, rapamycin. Cells were harvested 2 or 3 hours (h) after medium change. (A-D) ROS content was visualized with DCFDA and nuclei were stained with Hoechst fluorescent dye in control (A-B) and iodine deficient (C-D) cells (N=3). (E-F) 4-HNE adducts were detected using immunofluorescence after 3 h of ID (G) or control (F) treatment (N = 3). Representative pictures are shown. Scale bars = 20 µm. (G) VEGF mRNA expression was determined using...
RT-qPCR (N=6). (H) The involvement of ROS in ID-induced VEGF mRNA expression was studied by RT-qPCR thanks to the use of NAC (N=4). (I) The involvement of mTOR in ID-induced VEGF mRNA expression was studied by RT-qPCR thanks to the use of rapamycin (N=5). Data are expressed as means ± SEM. Statistical test: Unpaired Student's t test (G) or one-way ANOVA with Tukey-Kramer post-hoc test (H-I). *P < 0.05, **/+ P < 0.01, ***/+ + P < 0.001; *versus control, +versus ID 3h (G-I).

**Figure 7. Acute ID-induced blood perfusion increase in virgin mice is dependent on mTOR.** Virgin mice received an iodine containing diet (control) or iodine-deficient diet with 1% sodium perchlorate (ID) in the drinking water for 2 days (d) and were injected with either the vehicle solution (control and ID 2 days) or with rapamycin diluted in the vehicle solution (rapamycin and ID 2 days, rapamycin). Blood flow in mammary glands was measured with a Laser Doppler flowmeter (N=5). Data are expressed as means ± SEM. Statistical test: one-way ANOVA with Tukey-Kramer post-hoc test. *P < 0.05, **P < 0.01.

**Figure 8. Acute iodine deficiency influences NIS expression in MCF7 cells and in lactating mammary glands.** Lactating NMRI mice received an iodine containing diet (control) or iodine-deficient diet with 1% sodium perchlorate (ID) in the drinking water for 1 to 15 days (d) (A-B). MCF7 (C-D) and MCF12A (E-F) cells were cultured in iodine-containing medium for 7 days. Medium was replaced by iodine-containing (control) or iodine deficient (ID) medium. Cells were harvested 4 and 6 (MCF7) or 3 and 4 (MCF12A) hours (h) after medium change. (A, C, E) NIS mRNA expression was determined using RT-qPCR (A: control, ID 10 and 15 days: N=6, ID 1 and 4 days: N=5; C: N=4; E: N=3). (B, D, F) NIS protein expression was determined by western blotting (B: control, ID 1 and 4 days: N=5, ID 10 and 15 days: N=6; D: N=4; F: N=3). Data are expressed as means ± SEM. Statistical test: one-way ANOVA with Dunnett post-hoc test. *P < 0.05, versus control.
## Tables

### Table 1: antibodies used for western blotting

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<tr>
<th>Target protein</th>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Exposure time</th>
<th>Primary antibody: previous use</th>
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<tbody>
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<td>β-Actin</td>
<td>Monoclonal antibody, raised in mouse (Sigma #A5441)</td>
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<td>Goat anti-mouse-anti-HRP</td>
<td>30&quot; to 1'</td>
<td>(7; 8)</td>
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<td>HIF-1α (human)</td>
<td>Monoclonal antibody, raised in mouse (BD transduction #610959)</td>
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<td>(7; 17)</td>
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<tr>
<td>P70S6K</td>
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<td>Goat anti-rabbit-anti-HRP</td>
<td>2 to 4'</td>
<td>(7)</td>
</tr>
<tr>
<td>p(T389)P70S6K</td>
<td>Monoclonal antibody, raised in rabbit (Cell Signaling/Bioké #9234L)</td>
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<td>Goat anti-rabbit-anti-HRP</td>
<td>2 to 4'</td>
<td>(7)</td>
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Table 1: Primary and secondary antibodies and their dilution for western blot.

### Table 2: antibodies used for immunohistochemistry

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<th>Dilution</th>
<th>Secondary antibody</th>
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<td>VEGF-A</td>
<td>Monoclonal mouse anti-VEGF-A (Santa Cruz #SC-53462)</td>
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<td>Goat anti-mouse-anti-HRP, 1:50 (ThermosFisher Scientific #32230)</td>
<td>(7; 8; 17)</td>
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<tr>
<td>CD31</td>
<td>Polyclonal rabbit anti-CD31 (Abcam #ab28364)</td>
<td>1:100</td>
<td>Goat anti-rabbit-anti-HRP, 1:50 (thermosFisher Scientific #32260)</td>
<td>(10; 11)</td>
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Table 2: Primary and secondary antibodies and their dilution for immunohistochemistry.

### Table 3: primers used for qRT-PCR

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<th>Annealing temp.</th>
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<td>5’AGGAGGAGCAATGATCTTGAT3’</td>
<td>62°C</td>
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<tr>
<td>VEGF-A (mouse)</td>
<td>5’GAGTATATCTTCAAGCAGCTCTGTT3’</td>
<td>5’TTTCTTGCTTTTCTCATTTC3’</td>
<td>55°C</td>
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<tr>
<td>NIS (mouse)</td>
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<td>5’GGGCGGCGAGTCACATTCA3’</td>
<td>59°C</td>
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<tr>
<td>VEGF-A (human)</td>
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<td>5’CGGGAGAGGAGCTGAGCTGTA3’</td>
<td>62°C</td>
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<tr>
<td>NIS (human)</td>
<td>5’CCCCCCTCTGATTCCTGCTGTG3’</td>
<td>5’ACCAGCGCCCGACCTCTTCTTTATT3’</td>
<td>62°C</td>
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</table>

Table 3: Primer sequences and their annealing temperature for qRT-PCR.
A

B

C

D

E

F

G

H

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A

Control                           ID  4h

B                                       D
C                                      E

Control                               ID  4h

F                                             G

I

HIF-1α protein expression/β-actin

J

VEGF mRNA expression/β-actin **

K

VEGF mRNA expression/β-actin **

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Control | ID, 2h | Control
---|---|---
A | C | E
B | D | F

20 μm

G H I

**VEGF mRNA expression**/ actin

control | ID 3h | control | ID 3h | NAC | ID 3h NAC | control | ID 3h | Rapamycin | Rapamycin, ID 3h
---|---|---|---|---|---|---|---|---|---

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Mammary glands blood flow (% of control)

Control

ID 2d, rap

Amycin

ID 2d, rapamycin

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Lactating NMRI mice

NIS protein expression/\(\alpha\) actin

NIS mRNA expression/\(\alpha\) actin

MCF7

MCF12A

NIS protein expression/\(\alpha\) actin

NIS mRNA expression/\(\alpha\) actin

control ID 3h ID 4h

ID 10h

ID 15h

control ID 4h ID 6h

control ID 1d ID 4d ID 10d ID 15d

NIS mRNA expression/\(\alpha\) actin

NIS protein expression/\(\alpha\) actin

Lactating NMRI mice

MCF7

MCF12A