Inducible Nitric Oxide Synthase Is Involved in Endothelial Dysfunction of Mesenteric Small Arteries from Hypothyroid Rats

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The time-dependent effects of mild hypothyroidism on endothelial function were assessed in rat mesenteric arteries. Male Wistar rats were treated with methimazole (MMI; 0.003%) or placebo up to 16 wk. Endothelial function of mesenteric small arteries was assessed by pressurized myograph. MMI-treated animals displayed a decrease in serum thyroid hormones, an increment of plasma TSH and inflammatory cytokines, and a blunted vascular relaxation to acetylcholine, as compared with controls. Endothelial dysfunction resulted from a reduced nitric oxide (NO) availability caused by oxidative excess. Vascular-inducible NO synthase (iNOS) expression was up-regulated. S-methylisothiourea (an iNOS inhibitor) normalized endothelium-dependent relaxations and restored NO availability in arteries from 8-wk MMI-animals and partly ameliorated these alterations in 16-wk MMI rats. Similar results were obtained when MMI-induced hypothyroidism was prevented by T4 replacement. Among controls, an impaired NO availability, secondary to oxidative excess, occurred at 16 wk, and it was less pronounced than in age-matched MMI animals. Both endothelial dysfunction and oxidant excess secondary to aging were prevented by apocynin (nicotinamide adenine dinucleotide phosphate oxidase inhibitor). Mesenteric superoxide production was reduced by S-methylisothiourea and T4 replacement in MMI animals and abolished by apocynin in controls (dihydroethidium staining). MMI-induced mild hypothyroidism is associated with endothelial dysfunction caused by a reduced NO availability, secondary to oxidative excess. It is suggested that in this animal model, characterized by TSH elevation and low-grade inflammation, an increased expression and function of iNOS, resulting in superoxide generation, accounts for an impaired NO availability. (Endocrinology 150: 1033–1042, 2009)

Endothelium plays a major role in modulating vascular function and structure mainly by production and release of nitric oxide (NO), derived by the activity of endothelial NO synthase (eNOS) (1). Animal and human evidence have clearly documented that major cardiovascular risk factors, including aging, are characterized by the presence of endothelial dysfunction induced by an increased generation of reactive oxygen species (ROS), leading to NO breakdown (1, 2). Vascular nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase was recently identified as a major contributor to age-related ROS production in rat aorta (3). Other major sources of oxidative stress include the inducible NOS (iNOS) isofrom, which is greatly activated by low-grade vascular inflammation (4, 5). A dysfunctioning endothelium, characterized by reduced NO availability and increased ROS generation, is regarded as a crucial and early mechanism whereby low-grade vascular inflammation leads to atherosclerosis and increased risk of cardiovascular events (6, 7).

Hypothyroidism is a common endocrine disorder that affects nearly 10% of the general population (8). Growing evidence indicates that this clinical condition is associated with an increased risk of cardiovascular disease (9). Experimental studies reported that the hypothyroid status is characterized by a blunted endothelium-dependent relaxation in rat aorta and renal circulation (10–12). Moreover, a reduced vascular eNOS activ-

Abbreviations: ACh, Acetylcholine; DHE, dihydroethidium; E_max, maximal response; eNOS, endothelial nitric oxide synthase; EU, euthyroid; FT3, free T3; FT4, free T4; iNOS, inducible NO synthase; L-NAME, N^ω-nitro-L-arginine methyl ester; MMI, methimazole; NO, nitric oxide; NAD(P)H, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; SBP, systolic blood pressure; SMT, S-methylisothiourea.
ity was found in aorta from hypothyroid rats (13). However, these findings are in contrast with other studies, in which endo-
thelial dysfunction secondary to hypothyroidism was not de-
tected (12, 14). This discrepancy might be due to the different
duration and degree of hypothyroidism, an important aspect that
is likely involved in the pathogenesis of endothelial dysfunction
in such disease. Indeed, the impact of time-dependent exposure
of hypothyroidism on endothelial function and the underlying
mechanisms remain to be elucidated.

The present study was primarily designed to assess the time-
dependent influence of exposure to hypothyroidism on endo-
thelial function and the underlying mechanisms at the level of rat
mesenteric resistance arteries. For this purpose, we used rats with
hypothyroidism induced by low-dose methimazole (MMI), with
different duration of the disease, an experimental model that
mimics mild hypothyroidism in humans. In particular, we in-
vestigated whether a reduction in NO availability, ascribable to
an increased ROS generation, might play a role in the pathogen-
esis of endothelial dysfunction in this animal model. Because
aging can affect endothelial function per se, the impact of time-
dependent hypothyroidism was corrected for the worsening ef-
flect of aging. The secondary aim of our study was to assess the
contribution of vascular iNOS, NAD(P)H oxidase, and xanthine
oxidase as possible sources of ROS in this animal model of hy-
throidism. The effects of euthyroidism restored by adminis-
tration of T4 to MMI-treated rats were also evaluated.

Materials and Methods

Animal treatment

All experiments were carried out in accordance with the European
Male Wistar rats (Charles River, Lecco, Italy), initially weighing 220–
250 g (8–10 wk of age), were allocated to receive MMI (Teofarma, Pavia,
Italy; 0.003% in drinking water, MMI rats) or placebo
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Italy; 0.003% in drinking water, MMI rats) or placebo

Preparation and study of small arteries

Third-order branches of the mesenteric arterial tree were dissected
and placed in cold modified physiological salt solution, as previously
described (15, 16). Vessels were mounted on a pressurized myograph
and equilibrated [1 h with physiological salt solution, bubbled with 95%
air-5% CO2 (pH 7.4)] at 37 °C, as detailed elsewhere (15, 16).

Endothelium-dependent relaxation was assessed by measuring dia-
tory responses to cumulative concentrations of acetylcholine (Ach; 1
μM 100 μM Sigma Chemicals, St. Louis, MO). Endothelium-indepen-
dent relaxation was assessed by sodium nitroprusside (Sigma; 0.01–100
μM), a compound that acts directly on smooth muscle cells as an exog-
nenous NO donor (17). Vessels were precontracted with norepinephrine
(Sigma; 10 μM), whose concentration was chosen according to prelimi-
nary dose-titration experiments to establish the threshold concentration
able to elicit similar contractions among the experimental groups (data
not shown).

To evaluate NO availability and ROS production, concentra-
tion-response curves to Ach were constructed before and after 30 min pre-
incubation with the NO synthase inhibitor Nω-nitro-ω-arginine methyl
ester (L-NAME; 100 μM; Sigma) (18) or the antioxidant ascorbic acid
(Sigma; 10 mM, 30 min preincubation). To evaluate whether oxidative
stress could influence NO availability, an additional curve to Ach was
constructed under simultaneous incubation with L-NAME and ascorbic
acid. To evaluate the role of xanthine oxidase as a possible source of
ROS, curves to Ach were also obtained after 30 min preincubation with
the xanthine oxidase inhibitor allopurinol (Sigma; 100 μM).

Influence of iNOS on endothelium-dependent relaxation

To ascertain the contribution of iNOS, in further sets of mesenteric
vessels from 16-wk EU rats as well as 8- and 16-wk MMI animals,
concentration-response curves to Ach were constructed both before and
after 30 min incubation with the selective iNOS inhibitor S-methyliso-
thiourea (SMT; Sigma; 100 μM) (19). To evaluate whether impaired NO

| Table 1. Biological parameters from EU and MMI rats at each time point |
|---------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
|                                | 4-wk treatment | 8-wk treatment | 16-wk treatment | 8 wk | 16 wk |
| Animals (n)                    | EU | MMI | EU | MMI | EU | MMI | EU | MMI | EU | MMI | EU | MMI |
| Weight gain vs. baseline (g)   | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| TSH (ng/ml)                    | 4.5 ± 0.3 | 4.3 ± 0.4 | 10.4 ± 0.5 \(a\) | 4.1 ± 0.5 | 15.1 ± 0.7 \(a,d\) | 4.5 ± 0.7 | 16.8 ± 0.9 \(a,b\) | 4.1 ± 0.7 \(a,b\) | 4.3 ± 0.6 \(a,c\) |
| FT4 (pg/ml)                    | 18.1 ± 2.1 | 18.6 ± 2.3 | 11.7 ± 1.8 \(a\) | 19.1 ± 0.7 | 7.3 ± 1.1 \(a,d\) | 19.8 ± 1.0 | 6.4 ± 1.1 \(a,b\) | 20.5 ± 1.6 | 21.0 ± 1.4 |
| FT3 (pg/ml)                    | 4.8 ± 0.3 | 4.7 ± 0.2 | 4.7 ± 0.1 | 4.9 ± 0.3 | 3.3 ± 0.4 \(d\) | 4.5 ± 0.8 | 1.7 ± 0.1 \(a,b\) | 4.0 ± 0.3 \(a,b\) | 4.1 ± 0.3 \(a,c\) |
| TNFα (pg/ml)                   | 11.0 ± 0.7 | 12.3 ± 0.7 | 30.5 ± 1.4 \(a\) | 13.0 ± 1.5 | 49.1 ± 1.8 \(d\) | 15.8 ± 1.7 | 126.3 ± 3.7 \(b\) | 20.7 ± 3.8 | 19.2 ± 3.0 |
| IL-6 (pg/ml)                   | 73.5 ± 1.9 | 75.0 ± 1.2 | 116.5 ± 3.0 \(a\) | 76.1 ± 1.9 | 141.1 ± 3.0 \(d\) | 82.7 ± 2.1 | 166.5 ± 2.9 \(b\) | 79.2 ± 1.2 | 82.2 ± 2.9 \(c\) |
| SBP (mm Hg)                    | 106 ± 5 | 112 ± 6 | 110 ± 3 | 110 ± 4 | 109 ± 4 | 114 ± 5 | 113 ± 8 | 108 ± 2 | 110 ± 1 | 111 ± 5 |
| Heart rate (beats/min)         | 384 ± 7 | 384 ± 8 | 384 ± 6 | 391 ± 5 | 364 ± 5 \(a\) | 398 ± 10 | 347 ± 6 \(a\) | 378 ± 9 | 370 ± 11 \(c\) |
| Cholesterol (mg/dl)            | 74.5 ± 4.5 | 73.9 ± 3.6 | 68.1 ± 8.9 | 69.6 ± 8.4 | 73.8 ± 3.2 | 75.8 ± 4.2 | 75.1 ± 6.2 | 71.1 ± 9.2 | 75.8 ± 8.2 |

\(a\) P < 0.05 vs. EU.  
\(b\) P < 0.01 vs. 8-wk MMI.  
\(c\) P < 0.01 vs. 16-wk MMI.  
\(d\) P < 0.001 vs. 4-wk MMI.
availability was related to any biological effect exerted by iNOS, ACh was applied during simultaneous incubation with L-NAME and SMT.

**Role of NAD(P)H oxidase on endothelium-dependent relaxation**

To assess the role of NAD(P)H oxidase, additional 16-wk EU rats and 8- and 16-wk MMI rats were treated with the NAD(P)H oxidase inhibitor apocynin (Fluka, Buchs, Switzerland; 1.5 mM, drinking water) during the last 4 wk of the experimental period. This dose of apocynin was previously shown to prevent the vascular NAD(P)H oxidase activity (20). At the end of treatment, mesenteric vessels were isolated, and concentration-response curves to ACh were determined in the presence of L-NAME or ascorbic acid, as reported above.

**Effect of T4 replacement on endothelium-dependent relaxation in MMI-treated rats**

To assess the role of restored euthyroidism on MMI-induced endothelial dysfunction, an additional group of MMI-allocated animals received also injections of T4 (15 μg/kg/d sc, dissolved [1 μg/μl] in isotonic saline (100 ml) plus 1 ml 0.5 M NaOH). T4 treatment was started simultaneously with MMI intake. The dose of T4 was chosen to obtain a stable euthyroidism, as assessed by thyroid hormone assay. At the end of 8 and 16 wk of treatment, mesenteric vessels were isolated and concentration-response curves to ACh were obtained in the presence of L-NAME or SMT.

**Detection of superoxide anion**

The *in situ* production of superoxide anion was measured by means of the fluorescent dye dihydroethidium (DHE; Sigma), as previously described (21, 22). Briefly, unfixed frozen ring segments of mesenteric artery were cut into 30-μm-thick sections and placed on a glass slide. Three slides per segment were analyzed simultaneously after incubation with either SMT (100 μM), allopurinol (100 μM), or Krebs solution at 37 C for 30 min. Mesenteric artery rings from apocynin-treated rats were also assayed after incubation with SMT or Krebs solution. Krebs-HEPES buffer containing 2 μM DHE was applied onto each section, which was then coverslipped, placed in a dark humidified incubator at 37 C for 30 min, and evaluated under fluorescence microscopy. In the presence of superoxide, DHE is oxidized and it intercalates in cell DNA, thus staining the nucleus with red fluorescence (excitation at 488 nm, emission 610 nm). The percentage of arterial wall area stained with the red signal was then evaluated using an imaging computer software (McBiophotonics Image J; National Institutes of Health, Bethesda, MD).

**RNA extraction and real-time PCR**

This technique was used to assess mesenteric expression of mRNA for iNOS and eNOS isoforms. Total RNA was extracted by a RNeasy mini-kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. RNA was eluted with 35 μl of RNase-free water. Reverse transcription was performed using 1 μg of total RNA, random primers, and ImProm-II reverse transcriptase (Promega, Madison, WI) in a total volume of 20 μl. Real-time PCR was performed in the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The PCR mixture (25 μl) contained 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 μM of each deoxynucleotide triphosphate, iTaq DNA polymerase 50 U/ml, 6 mM MgCl2, SYBR green I, 20 μM fluorescent, and an appropriate volume of cDNA preparation. The PCR cycling conditions included a 95 C heating step for 15 sec, annealing at 59–60 C for 45 sec, and extension at 55 C for 30 sec. Primer sequences used were: rat iNOS, 5′-TGGCTCTC-CCTCTGGAAAAG-3′ (forward), 5′-GGTGGTCATGATGTCATGTCAT-3′ (reverse); eNOS, 5′-CACACTGCTAGGTTGCGGAA-3′ (forward), 5′-TGCTGACGGCTGAGTAGTA-3′ (reverse); ß-actin, 5′-TTGCTGTACCATCTGTCGG-3′ (forward), 5′-GAGACATGTCAGAAGAGAT-3′ (reverse). The data generated from SYBR green were analyzed using Gene Expression Macro (version 1.1; mathematical model Jo Vandesompele; Bio-Rad).

**Western blot assay**

Protein expression of iNOS and eNOS in specimens of mesenteric arteries was assessed by Western blot assay. Samples were homogenized in lysis buffer m (pH 7.4), NaCl 150 mM, EDTA 0.2 mM, phenylmethylsulfonyl fluoride 2 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml, sodium fluoride 1 mM, sodium orthovanadate 1 mM, glycerol 2%, MgCl2 0.5 mM, Triton X-100 1%). The lysates were centrifuged at 20,000 × g for 15 min at 4 C for 30 min, and supernatants were stored at −80 C. Protein concentration was determined by Bradford method (Bio-Rad, Hercules, CA). Equivalent amounts of protein lysates (50 μg) were separated by electrophoresis on sodium dodecylsulfate polyacrylamide gel (8%) and transferred onto a nitrocellulose membrane. Blots were blocked overnight with 5% nonfat dried milk in PBS and incubated overnight at room temperature with mouse monoclonal anti-iNOS or anti-eNOS (BD Biosciences, San Diego, CA). After washing with 0.1% Tween 20 in Tris-buffered saline, a peroxidase-conjugated rabbit antimouse antibody was added (1 h, room temperature). After washing with 0.1% Tween 20 in Tris-buffered saline, immunoreactive bands were visualized by incubation with chemiluminescent reagents and exposed to Image Station 440 (Kodak, Rochester, NY) for signal detection and densitometric analysis. Blots were then stripped and reprobed with anti-ß-actin antibody. The relative intensity of immunoreactive bands was normalized to that of ß-actin.

**Serum thyroid hormones, cytokine, and cholesterol assays**

After collection, blood samples were immediately placed in heparinized tubes. Serum was separated by centrifugation and stored at −70 C until assayed. Specific RIAs were used to measure serum FT3 and FT4 (DiaSorin, Rome, Italy) or TSH levels (Biocode-Hycel, Liege, Belgium). TNF-α and IL-6 were assayed by ELISA commercial kits (R&D Systems, Minneapolis, MN), whereas total serum cholesterol by enzymatic method (Roche, Mannheim, Germany).

**Data analysis**

Maximal ACh- and sodium nitroprusside-induced responses (E_{max}) were calculated as maximal percentage increments of lumen diameter. Results are presented as mean ± SEM. The number (n) of rats is indicated in Table 1. The statistical significance of relaxation responses was assessed taking into consideration time course and treatment by two-way ANOVA. Other comparisons were made by repeated-measures ANOVA or one-way ANOVA followed by a Student Newman-Keuls test, where appropriate. P < 0.05 was considered statistically significant.

**Results**

**Biological parameters**

At wk 4, MMI rats showed a trend toward a reduced gain of body weight, doubled TSH levels, and decreased FT4, but still normal FT3, values compared with EU rats (Table 1). At wk 8, MMI rats showed a lower body weight, a further TSH increase, concomitantly reduced levels of FT3, and FT4, and a decreased heart rate compared with age-matched EU controls. These differences were more evident after 16 wk of MMI administration (Table 1). In MMI rats, but not in EU groups, a significantly progressive increase in TNF-α and IL-6 serum levels was observed. SBP and cholesterol levels were similar in all groups at any time.

**Endothelium-dependent relaxation in EU and MMI rats**

In EU animals, relaxation to ACh was preserved up to wk 8, but it was found to be attenuated at wk 16 (Fig. 1A). In MMI rats,
relaxation to ACh was not altered at wk 4, but it was significantly reduced at wk 8 and further declined at wk 16 (Fig. 1B). At wk 16, vessels from MMI rats showed a greater reduction in the response to ACh compared with age-matched EU rats (ACh decrement above baseline: MMI, 30.6 ± 1.7%; EU, 10.0 ± 0.9%; P < 0.001). Relaxation to sodium nitroprusside was similar between EU and MMI rats as well as among the subgroups evaluated at different time points (data not shown).

**Effects of L-NAME, ascorbic acid, and allopurinol on endothelium-dependent relaxation**

At baseline, relaxation to ACh was significantly blunted by L-NAME and unmodified by ascorbic acid. In EU rats, the inhibitory effect of L-NAME on the response to ACh was preserved and not modified by advanced age until 16 wk of placebo treatment, when a reduced inhibition by L-NAME on ACh was observed (Fig. 1C). Analogously, ascorbic acid, although ineffective in younger subgroups, normalized the relaxation to ACh and restored the inhibitory effect of L-NAME on ACh at wk 16 (Fig. 1C). In the 16-wk EU group, the blunted response to ACh was unaffected by allopurinol (data not shown).

In MMI animals, the inhibition of L-NAME on ACh started to decline after 8 wk of MMI intake and decreased further in the 16-wk subgroup, in which the response to ACh was virtually unaffected by L-NAME (Fig. 1D). Ascorbic acid normalized the relaxation to ACh and restored the inhibitory effect of L-NAME on ACh both in 8- and 16-wk MMI groups (Fig. 1D). Allopurinol failed to affect the responses to ACh, in both the 8- and 16-wk groups (data not shown).

**Influence of iNOS on endothelium-dependent relaxation**

In vessels from 16-wk EU rats, the attenuated relaxation to ACh and the inhibition by L-NAME on ACh were not affected by SMT administration (E_max ACh: 85.0 ± 1.0%; ACh+L-NAME: 55.2 ± 1.7%; ACh+SMT: 85.8 ± 0.7%; ACh+L-NAME+SMT: 55.8 ± 0.9%; P = NS) (Fig. 2). Different results emerged from MMI animals. Indeed, in the 8-wk group, the relaxation to ACh and the inhibition of L-NAME on ACh were normalized by SMT (E_max ACh: 78.5 ± 0.7%; ACh+L-NAME: 66.3 ± 0.5%; ACh+SMT: 97.7 ± 0.3%; ACh+L-NAME+SMT: 59.8 ± 0.9%; P < 0.001) (Fig. 2). In contrast, in the 16-wk group, SMT greatly improved, but did not normalize, the relaxation to ACh and partly ameliorated the

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**FIG. 1.** Upper panels, Relaxations to ACh in mesenteric arteries at baseline and from EU- (A) or MMI-treated (B) rats at different time points. Each point represents the mean of six experiments ± SEM. *, P < 0.05; †, P < 0.01; ‡, P < 0.001. Lower panels, Inhibitory effect of L-NAME (100 μM) on ACh-induced maximal relaxation ± ascorbic acid (Asc. Ac; 10 mM) in mesenteric arteries from EU (C) or MMI rats (D). Each column represents the mean of six experiments ± SEM. *, P < 0.05 vs. other groups; †, P < 0.001 vs. 4 w; ‡, P < 0.01 vs. 8 wk. w, Weeks.
inhibition by L-NAME on ACh (E_max: 64.4 ± 1.9%; ACh+L-NAME: 58.7 ± 2.1%; ACh+L-NAME+SMT: 58.7 ± 0.8%; P < 0.01 vs. baseline, Fig. 2).

Role of NAD(P)H oxidase inhibition on endothelium-dependent relaxation

The age-related endothelial dysfunction was prevented by apocynin. Thus, in 16-wk EU apocynin-treated rats, relaxation to ACh was preserved (E_max: 97.5 ± 0.7%), significantly blunted by L-NAME (inhibition: -40.1 ± 0.5%) and no longer affected by ascorbic acid (E_max: 96.0 ± 0.6%). Opposite results emerged from MMI animals. Indeed, in the 8-wk group, apocynin did not affect relaxation to ACh (E_max: 79.6 ± 0.9%) and the blunting effect of L-NAME on ACh (inhibition, -12.8 ± 1.3; P = NS vs. 8 wk MMI rats). In the 16-wk group, apocynin significantly improved, but did not normalize, relaxation to ACh (E_max: 83.0 ± 0.7%) and partly ameliorated the inhibition by L-NAME on ACh (inhibition, -26.1 ± 1.8; P < 0.01 vs. baseline). Ascorbic acid normalized the relaxation to ACh in both the 8- and 16-wk groups (data not shown).

Effect of T4 replacement on endothelium-dependent relaxation in MMI-treated rats

T4 administration prevented hypothyroidism, in both the 8- and 16-wk MMI groups, as indicated by the normal serum thyroid hormone pattern (Table 1). Serum cytokine concentrations were also normalized (Table 1). In the 8-wk MMI group, T4 replacement normalized the relaxation to ACh and restored the inhibition of L-NAME on ACh (E_max: 94.3 ± 0.6%; ACh+L-NAME: 53.6 ± 0.7%; P < 0.001 vs. 8-wk MMI, Fig. 2). SMT failed to affect the relaxation to ACh as well as the inhibition by L-NAME on ACh (E_max: 95.6 ± 0.6%; ACh+L-NAME+SMT: 55.1 ± 1.0%; P = NS vs. saline, Fig. 2). In contrast, in the 16-wk group, T4 greatly improved, without normalizing, the relaxation to ACh and partly ameliorated the inhibition by L-NAME on ACh (E_max: 87.8 ± 0.8%; ACh+L-NAME: 59.4 ± 2.0%; P < 0.01 vs. 8-wk MMI, Fig. 2). In this group, SMT failed to modulate either the relaxation to ACh or the inhibitory effect of L-NAME on ACh (E_max: 61.0 ± 2.9%; P = NS vs. saline, Fig. 2).

DHE analysis of superoxide anion generation

In EU rats, DHE red fluorescence revealed a significant increase in mesenteric superoxide anion production only in the 16-wk group compared with baseline (Fig. 3). In MMI rats, an increased superoxide anion production was already present in the 8-wk group, and it was further enhanced at 16 wk (Fig. 3). Of note, at this time the superoxide anion production was much greater in MMI compared with EU vessels. The concomitant MMI-T4 administration completely abrogated the superoxide production at 8 wk and promoted only a partial reduction in the 16-wk group (Fig. 3).

Among 16-wk EU vessels, the superoxide production was not affected by incubation with SMT, whereas it was completely prevented by apocynin (Fig. 4). Among 16-wk MMI rats, the superoxide production was partly reduced by either SMT or apocynin treatment (Fig. 4). In this group, superoxide production was completely abrogated only when SMT and apocynin were simultaneously present (Fig. 4). Incubation with allopurinol was devoid of any effect in any group at any time (data not shown).

RT-PCR and Western blot analysis of iNOS and eNOS isoforms

RT-PCR showed a weak expression of mRNA encoding iNOS at baseline, with no significant variations in EU groups throughout the observational period. In contrast, among MMI animals, a significant induction of iNOS emerged in the 8-wk group, with a further enhancement at 16 wk (Fig. 5A). T4 replacement completely prevented the MMI-induced iNOS expression (Fig. 5A).

Western blot analysis detected a slight basal iNOS expression, with a significant increase in EU rats at 16 wk. In MMI animals, iNOS showed an increment at wk 8, with a greater enhancement...
at wk 16 (Fig. 5B). This effect was prevented by T4 administration (Fig. 5B).

eNOS mRNA expression and protein levels, assessed by RT-PCR and Western blot, respectively, did not significantly vary in both EU and MMI rats at any time (Fig. 5, C and D). Such patterns were not affected by T4 treatment (Fig. 5C, D).

Discussion

The first major novel finding of the present study consists in the demonstration of endothelial dysfunction in mild hypothyroid rats and the identification of underlying mechanisms. We found that mesenteric small arteries from animals treated for 8 wk with MMI showed a blunted endothelium-dependent relaxation, together with a reduced inhibitory effect of L-NAME on acetylcholine, compared with age-matched controls. These alterations were more pronounced after longer (16 wk) exposure to MMI, and they were completely prevented by ascorbic acid at each time. Accordingly, DHE analysis revealed an increased vascular superoxide anion generation starting after 8 wk of MMI treatment, with a dramatic increment at 16 wk. Taken together, these findings provide the first demonstration that exposure to mild hypothyroidism induces a progressive endothelial dysfunction at the level of resistance vasculature and that such alteration depends on a reduced NO availability secondary to increased vascular ROS production. To exclude the possibility that these effects might be caused by ageing per se, a well-recognized major contributor to vascular functional changes (1, 2), results from each time of exposure to MMI were compared with corresponding placebo groups. Indeed, vessels from the 8-wk placebo group showed a normal endothelium-dependent relaxation, together with a preserved inhibitory effect of L-NAME and no evidence of ROS generation. At wk 16, the placebo group displayed an endothelial dysfunction due to a reduced NO availability secondary to increased ROS production. These alterations appeared later (wk 16 vs. wk 8) and were significantly less pronounced compared with MMI-treated animals. Therefore, these data, whereas extending to resistance vasculature previous demonstration that ROS excess accounts for age-related vascular functional changes (3), indicate that endothelial dysfunction secondary to mild hypothyroidism occurs independently from aging.

The impact of hypothyroidism on vascular endothelial function has been previously addressed in a different rat model of hypothyroidism, consisting of severe propylthiouracil-induced hypothyroidism, without conclusive results. In this model, when conductance arteries were examined, some authors described a blunted endothelium-dependent relaxation (10, 11), whereas others failed to confirm this finding (12). Conflicting data have
been also obtained from vascular resistance districts, in which a blunted (12) or a preserved (14) endothelial function was described. Different degrees of hypothyroidism or differences in the vessel preparation used might account for these discrepancies. Nevertheless, our study is the first to indicate that exposure to mild hypothyroidism induces a progressive condition of endothelial dysfunction in resistance small arteries.

Our experimental conditions did not allow to detect the exact pathogenetic mechanism whereby hypothyroidism induces endothelial dysfunction, being high TSH levels and low thyroid hormone concentrations, together with low-grade inflammation, simultaneously present in our experimental model. With respect to a possible direct role of TSH, the early increment (4 wk) of TSH values, together with an increment of serum cytokines in MMI rats, was not accompanied by any endothelial dysfunction, which emerged only at wk 8 of MMI treatment. Although it is possible that elevated TSH and low-grade inflammation require additional time to induce endothelial dysfunction, the specific role of elevated TSH as a mechanism accounting for endothelial dysfunction in our study cannot be demonstrated.

In a previous report, an endothelial dysfunction was demonstrated in aortas taken from a murine model of thyroid hormone resistance, characterized by elevated TSH (23). In vitro studies reported that TSH, through a cAMP-protein kinase A pathway, is able to induce low-grade inflammation (24), a condition greatly involved in the pathogenesis of endothelial dysfunction (5, 6, 25). In humans, in whom functional TSH receptors have been documented in endothelial cells (26), we recently described low-grade systemic inflammation as a cause of endothelial dysfunction in patients with elevated TSH levels but normal free thyroid hormones (27). However, when recombinant human TSH was intrabrachially infused, an enhancement of forearm endothelial function was reported (28).

With respect to a possible direct role of thyroid hormones on endothelial function, in a previous study, Bussemaker et al. (29) showed that chronic T3 application was able to enhance endothelium-dependent relaxation in rat renal arteries, together with eNOS up-regulation in aorta. This finding indirectly suggests that in our study low T3 concentrations, detectable at 8 wk of MMI intake, play a role in endothelial dysfunction. Of note, our results indicate that low T3 levels were not associated with any decrement of vascular eNOS expression, a finding likely due to the short period of overt hypothyroidism in our animal model. However, the impact of thyroid hormones on vascular eNOS expression is still controversial because in another report, Grieve et al. (30) documented an aortic eNOS up-regulation in the pres-

FIG. 4. DHE staining for detection of superoxide generation. Representative DHE staining (upper panel) and quantitative analysis of the red signal (lower panel, magnification, ×40) in mesenteric arteries from EU or MMI rats at 16 wk of exposure ± apocynin and SMT. Each column represents the mean of four to six experiments ± SEM. * P < 0.05; † P < 0.001.
ence of an opposite pathological condition, such as the propyl-thiouracil-induced hypothyroidism.

Our experiments with concomitant MMI and T4 replacement, although indicating a complete restoration of endothelial function and NO availability at 8 wk, documented only a partial amelioration in the 16-wk group, thus confirming the persistence of a parallel aging-related component of endothelial dysfunction in such animals. Of note, T4 replacement led to a concomitant normalization of TSH and cytokine serum levels, a condition that does not allow to discriminate on a predominant role of each parameter in determining endothelial dysfunction. Whatever the exact mechanism implicated in determining functional alterations, it appears clear that in our study endothelial dysfunction was first detected after 8 wk of treatment when thyroid hormones were significantly reduced. Therefore, taken together, our findings allow us to state that low thyroid hormones, together with elevated TSH and low-grade inflammation, contribute to endothelial dysfunction. Moreover, our results from MMI plus T4 treatment argue against the possibility that MMI per se might exert a direct toxic effect on endothelial cells.

The second major novel finding of the present study is the demonstration of a vascular source of oxidative stress in mild hypothyroid rats. The role of iNOS, an enzyme pathway with recognized implications in ROS production, particularly under inflammatory conditions (25, 31), was addressed by testing the selective inhibitor SMT. The choice to investigate iNOS was supported by the observation that low-grade inflammation, which characterizes MMI rats, is a well-demonstrated stimulus for iNOS induction (4, 5, 32). We also tested apocynin, a specific inhibitor of NAD(P)H oxidase, because of the major role played by this enzyme in age-related ROS generation (3). In addition, the role of xanthine oxidase, regarded as a major endothelial source of superoxide (33), was assessed by allopurinol. At 8 wk of MMI administration, a significant iNOS induction, together with an increased superoxide production, was detected. Moreover, iNOS blockade, but not apocynin, normalized the endothelium-dependent relaxation and restored the NO availability, up to values similar to those obtained by ascorbic acid application. Opposite findings emerged from control EU animals because in the 16-wk placebo-treated group, SMT was without effects.
whereas apocynin prevented endothelial dysfunction. Thus, these data suggest that iNOS is implicated in endothelial dysfunction in hypothyroid animals, whereas NAD(P)H oxidase is implicated in age-related functional alterations. Results from 16-wk MMI-treated animals support this concept. Indeed, apocynin restored only in part the impaired endothelial function. Similarly, SMT ameliorated, but did not normalize, the NO availability.

Our findings from DHE staining, although corroborating the functional results, strongly support distinct roles of each source in determining superoxide production in this animal model. Among aged controls, SMT failed to affect superoxide generation, whereas apocynin completely prevented it. Moreover, among MMI rats, SMT or apocynin reduced superoxide generation at 16 wk. Of note, in these animals, the increased superoxide generation was abolished only when SMT and apocynin were simultaneously present. Moreover, no increased plasma levels of inflammatory cytokines were observed in placebo-treated animals at any time, a finding that further supports the independence of the two mechanisms leading to increased ROS in mild hypothyroidism and aging. Taken together, these data demonstrate that in MMI rats, but not controls, iNOS is a main source of oxidative stress, whereas they confirm the role of vascular NAD(P)H oxidase as a source of ROS in aging (3). Vascular superoxide detection among MMI-animals treated with T₄ further strengthens our view. Thus, in the 8-wk MMI group, when age-related ROS production was still not evident, superoxide generation was virtually abolished, whereas in the 16-wk group, concomitant T₄ was partly able to prevent superoxide generation. Finally, the negative results obtained with allopurinol argue against a major role of xanthine oxidase, at least under our conditions. Although the fine mechanism responsible for iNOS induction was beyond the scope of our study, the finding obtained from the 4-wk MMI group, showing elevated TSH levels despite concomitant T₄, seems to exclude TSH as a major factor regulating iNOS.

In conclusion, the present study indicates that MMI-induced mild hypothyroidism progressively induces endothelial dysfunction caused by a reduced NO availability secondary to an enhanced oxidative stress. It is suggested that in this animal model, characterized by decreased T₄, TSH elevation and low-grade inflammation, an increased expression and function of iNOS, resulting in superoxide generation, accounts for an impaired NO availability.

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