Attenuated PGI2 synthesis in obese Zucker rats

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Hodnett BL, Dearman JA, Carter CB, Hester RL. Attenuated PGI2 synthesis in obese Zucker rats. Am J Physiol Regul Integr Comp Physiol 296: R715–R721, 2009. First published December 31, 2008; doi:10.1152/ajpregu.90330.2008.—In obesity, skeletal muscle blood flow during exercise (functional hyperemia) is impaired. We have indirectly demonstrated that an altered arachidonic acid metabolism is responsible for the impaired functional vasodilation in the obese Zucker rat (OZR), a model of obesity. In this study, we tested the hypothesis that there is an impaired release of PGI2 due to a nitration of PGI2 synthase (PGIS), which is associated with a decreased prostanooid receptor expression. PGI2, PGE2, and thromboxane A2 (TXA2) release were determined in vitro using ELISA under basal conditions and in response to arachidonic acid (AA) administration (50 μM). Immunofluorescence of PGI2 and TXA2 receptors (IP and TP, respectively) was determined in dispersed vascular smooth muscle cells (VSMCs). Nitration of tyrosine residues of the PGIS enzyme was determined using immunoprecipitation and Western blot analysis. Following AA administration, PGI2 and PGE2 release were attenuated in OZR compared with lean Zucker rats (LZR; controls). Basal and AA-induced TXA2 release were not significantly different between groups. IP and TP immunofluorescence were not significantly different between OZR and LZR groups. OZR exhibited elevated nitration of tyrosine residues of PGIS compared with LZR. These results suggest that alterations in the PGI2 pathway (attenuated PGI2 synthesis), and not the TXA2 pathway (normal TXA2 synthesis/no change in TP receptor expression), underlie the attenuated functional hyperemia in the OZR.

vasodilation; arachidonic acid; microcirculation; exercise

OBESITY IS A METABOLIC DISORDER associated with insulin resistance, hyperglycemia, dyslipidemia, a proinflammatory state, elevations in oxidative stress, and endothelial dysfunction. Obesity itself is a risk factor for cardiovascular diseases including hypertension, coronary artery disease, and stroke, as well as other metabolic disorders, such as type II diabetes and metabolic syndrome (1). One treatment option for obesity that has been shown to improve weight control is exercise. However, obesity has been shown to limit the normal increase in muscle blood flow during exercise (functional hyperemia) in both human (20, 27) and animal models (7, 41, 42); thus, limiting the benefit of exercise as an effective treatment option for obese patients.

The exact mechanisms underlying the impaired functional hyperemia in obesity are unclear. One potential cause of the impaired blood flow response is obesity-induced endothelial dysfunction resulting in attenuated release of endothelium-derived relaxing factors, several of which have been shown to regulate arteriolar diameter and muscle blood flow during exercise (34). These endothelium-derived relaxing factors include the prostaglandins, metabolites of arachidonic acid (AA), and the cyclooxygenase pathway (11, 19, 23). Prostacyclin (PGI2) is the primary prostaglandin released from endothelial cells and is considered to be involved in the functional hyperemic response (32, 33). Exercise has been shown to be associated with an increase in PGI2 levels, whereas inhibition of prostaglandin synthesis has been shown to markedly attenuate functional hyperemia (28, 38, 39). PGI2 results in vasodilation through activation of the PGI2 receptor (IP) (14, 31), and stimulation of the IP receptor using PGI2 analogs has been shown to result in an attenuated vasodilation in the obese Zucker rat (OZR), both under in vivo (42) and in vitro (7) experimental conditions. The mechanisms underlying the impaired IP receptor-mediated vasodilation in obesity are unclear and may be due to alterations in downstream signaling and effector mechanisms. However, it is unknown whether PGI2 synthesis itself is impaired in the OZR skeletal muscle vasculature, which would potentially result in diminished stimulation of the IP receptor, and an overall decrease in IP receptor-mediated vasodilation.

Another potential mechanism for the impaired functional hyperemic response in the OZR is an increase in production of the vasoconstrictive metabolite thromboxane A2 (TXA2), which could result in an enhanced TXA2 receptor (TP) stimulation, resulting in an enhanced vasoconstriction that might limit vasodilation in response to other mechanisms. It is unclear to what extent TXA2 production is altered in the OZR, as the TP receptor may also be stimulated by the precursor product of both PGI2 and TXA2, which is PGH2 (29, 30).

The aim of this study was to test the hypothesis that normal AA metabolism is altered in the OZR via a decrease in PGI2 synthesis and an increase in TXA2 production. We also examined whether the synthesis of another prostaglandin, PGE2, was impaired in the OZR, to assess whether prostaglandin production in general might be impacted. PGE2 has also been shown to be released following exercise in humans (16, 17, 39), although its role in the control of skeletal muscle blood flow in rats, much less in obese models, is largely unknown. In addition to measuring these AA metabolites, we determined whether alterations in IP and TP receptor immunofluorescence of isolated vascular smooth muscle cells accompanied the altered AA metabolism. Finally, we measured nitration of tyrosine residues of PGI2 synthase (PGIS), a mechanism by which diminished PGI2 synthesis can occur.

MATERIALS AND METHODS

Animals. The experimental protocols for this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Male lean Zucker rats
(LZR: \(n = 17; 349 \pm 6\) g) and OZR (\(n = 14; 522 \pm 12\) g) (11-to-14-wk-old; Harlan, Indianapolis, IN) were used for these experiments. All animals were housed two to three animals per cage at 22°C (12:12-h light-dark cycle) with ad libitum access to food and water. All animals were anesthetized with pentobarbital sodium (65 mg/kg ip) prior to surgery. Following tissue removal, all animals were euthanized by an overdose of pentobarbital sodium. Death was confirmed by a lack of a heartbeat and spontaneous breathing.

Prostanoid synthesis assay. Both left and right femoral arteries were removed from LZRs and OZRs and placed in a Silastic-coated petri dish containing dissection solution (in mM: 130 NaCl, 4 KCl, 1.2 MgSO4, 4 NaHCO3, 1.8 CaCl2, 10 HEPES, 1.18 KH2PO4, 0.03 EDTA, 6 glucose). The arteries from LZR (\(n = 10\)) and OZR (\(n = 7\)) were cut into equal length (4-mm) segments, placed in wells of a 96-well plate containing a buffer solution (in mM: 118.07 NaCl, 6.17 KCl, 2.55 CaCl2, 25 NaHCO3, 5.5 glucose), and equilibrated in a tissue culture incubator for 1 h. To determine basal prostanoid release, the arteries were removed, placed in fresh buffer, and maintained for 3 h at 37°C (5% CO2-balance air). The buffer was removed for analysis and replaced with fresh buffer containing 50 \(\mu\)M AA to determine alterations in stimulated prostanoid release. The arteries were incubated for an additional 3 h in the incubator, following which the samples were collected and stored at −20°C until analysis. For each animal, the samples were averaged from both femoral arteries to make one data point. PGI2 and TXA2 in the buffer were detected by the presence of their stable metabolites, 6-keto-PGF1\(_\alpha\) and TXB2, respectively, using commercially available ELISA kits (Neogen, Lexington, KY). PGE2 was detected directly using an ELISA kit (Neogen). The above chemicals were purchased from Sigma (St. Louis, MO).

IP and TP immunofluorescence. Arterioles were dissected from the gracilis muscles of LZR (\(n = 3\)) and OZR (\(n = 3\)) animals and placed in dissection solution. Vascular smooth muscle cells (VSMCs) were dispersed via enzymatic digestion in HBSS containing 26 U/ml papain and 1 mg/ml dithioerythritol for 15 min at 37°C, followed by incubation in HBSS containing 2 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 75 U/ml elastase for 12 min at 37°C. Following the enzymatic digestion, vessels were rinsed with HBSS and triturated to dissociate VSMCs. Cells were then filtered and examined using a light microscope to check for dispersion. Dissociated VSMCs were fixed in 4% paraformaldehyde, plated on glass slides, and dried for ~2 h at 37°C. Following drying, samples were rehydrated and washed in PBS and then blocked with 5% normal donkey serum in PBS for 1 h before the addition of primary antibodies. VSMCs were incubated overnight at 4°C with a mouse monoclonal antibody for \(\alpha\)-actin (1:200 dilution; Sigma) and a polyclonal antibody for one of the receptors: rabbit anti-IP receptor or rabbit anti-TP receptor (1:500 dilution; Cayman, Ann Arbor, MI). Samples where then washed with PBS and incubated with the fluorescent secondary antibodies donkey anti-mouse DyLight 488 and donkey anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) with all "above chemicals were purchased from Sigma (St. Louis, MO).

Statistics and data analysis. Prostanoid release data were analyzed using two-way repeated-measures ANOVA. The ratio of nitrotyrosine to total PGIS was used as an index of nitration.

RESULTS

Prostanoid release. Basal PGI2 release was not significantly different between LZR and OZR (Fig. 1). AA administration induced a significant increase in PGI2 production in both groups (\(P < 0.001\) for LZR and OZR). However, the stimulated PGI2 release in response to AA was significantly impaired in the OZR compared with the LZR (\(P = 0.049\)). Basal and AA-induced TXA2 release were not significantly different between the two groups (Fig. 2). In addition, the

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\text{spin columns for 4 h at 4°C. Protein G agarose beads were added to the cell lysate, incubated, and mixed overnight at 4°C. The beads were then washed, and the precipitated proteins were collected by elution with 1X Laemmli buffer (Sigma). Equal amounts of protein were then separated using SDS-PAGE (4–20% gradient Tris–HCl gels; Jule, Milford, CT) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked for 1 h in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and incubated overnight at 4°C with rabbit anti-PGIS (1:500 dilution). For immunochromal labeling of PGIS, blots were incubated for 1 h at room temperature with a fluorescent secondary antibody (goat anti-rabbit IgG; 1:5,000 dilution; Rockland Immunochemicals, Gilbertsville, PA). Following immunochromal labeling of PGIS, the bands were detected and quantified for total PGIS by using an Odyssey infrared imaging system (LI-COR Biosciences). Following PGIS detection, blots were then washed in PBS containing 0.1% Tween-20 and incubated overnight at 4°C with mouse antinitrotyrosine (1:200 dilution; Santa Cruz Biotechnology). For immunochromal labeling of nitrotyrosine, blots were then incubated for 1 h at room temperature with donkey anti-mouse IgG (1:2,000 dilution; Abcam, Cambridge, MA). Following the second immunochromal labeling of the PGIS bands, blots were rescanned to detect and quantify nitrotyrosine residues. The quantification of the PGIS bands for total PGIS and for nitrated PGIS was performed using densitometric analysis of scanned images using Odyssey 1.1 software (LI-COR Biosciences). The ratio of nitrated PGIS to total PGIS was used as an index of nitration.

Statistics and data analysis. Prostanoid release data were analyzed using two-way repeated-measures ANOVA. The Student-Newman-Keuls post hoc test was used to compare individual groups. Immunofluorescence and nitration data were analyzed using Student’s t-test. A probability of \(P \leq 0.05\) was accepted as statistically significant for all comparisons. All data are presented as means ± SE.

Fig. 1. Basal and arachidonic acid (AA)-induced PGI2 release from femoral arteries of lean (\(n = 10\)) and obese (\(n = 7\)) Zucker rats. Values are means ± SE. *Significant difference between basal and arachidonic acid-induced PGI2 release; #significant difference between lean and obese AA-induced PGI2 release.
difference between the basal and AA-induced levels within a
group only reached significance for the LZR (P = 0.002).
PGE2 results were similar to those for PGI2 (Fig. 3). AA
induced significant increases in PGE2 synthesis in both groups
(P < 0.001 for LZR and OZR). Basal PGE2 release was not
significantly different between groups; whereas, AA-induced
PGE2 levels were significantly lower in OZR as compared with
LZR (P = 0.019).

IP and TP receptor immunofluorescence. IP receptor immu-
nofluorescence in VSMCs was similar between the LZR (n =
3 animals) compared with the OZR (Fig. 4; n = 3 animals).
Likewise, TP receptor immunofluorescence in VSMCs was
similar between the LZR (n = 3 animals) and OZR (n = 3
animals) groups (Fig. 5).

Nitration of PGIS. Following immunoprecipitation of the
prostacyclin synthase enzyme from thoracic aorta homogenates
of lean and obese animals, Western blot analyses were used to
detect the prostacyclin synthase enzyme itself and nitrated
tyrosine residues. Prostacyclin synthase was detected as two
sets of bands, an upper band at ~50 kDa and a lower band at
~30 kDa. For each band, the ratio of nitrated PGIS to total
PGIS was quantified. There was no significant difference in
nitrotyrosine/PGIS of the upper band between LZR and OZR
(Fig. 6A). Nitrotyrosine/PGIS of the lower band (Fig. 6B) was
significantly elevated in the OZR compared with the LZR (P =
0.027).

DISCUSSION

The major findings of this study are that 1) PGI2 synthesis is
impaired in the vasculature of OZRs, 2) TXA2 production is
not elevated in the OZR, 3) PGE2 synthesis is also impaired in
the OZR, 4) IP receptor immunofluorescence is similar be-
between LZR and OZR VSMCs, 5) TP receptor immunofluores-
cence is not elevated in OZR VSMCs, and 6) nitration of
tyrosine residues of prostacyclin synthase is significantly ele-
Vated in the OZR vasculature. These findings support our
hypothesis that the impaired functional hyperemia in the OZR
may be due to alterations in normal AA metabolism resulting
in impaired PGI2-mediated vasodilation. This altered AA
mechanism appears to be due to alterations in the PGI2-
mediated pathway with no alterations in the TXA2-mediated
pathway. Diminished production of PGI2 is likely due to
increased nitration of tyrosine residues of the PGIS enzyme
responsible for PGI2 synthesis.

The OZR, a model of obesity similar to humans, exhibits
insulin resistance, hyperglycemia, dyslipidemia, endothelial
dysfunction, and increased oxidative stress (9, 25, 41). In
addition, this model of obesity exhibits significantly impaired
functional hyperemia (7, 41, 42). Several mechanisms are
thought to play a role in the altered functional hyperemia seen
in the OZR, including structural remodeling of the microcirculation (5, 8), alterations in nitric oxide- (10) and AA-induced vasodilatory responses mechanisms (6, 42), enhanced \( \alpha \)-adrenergic reactivity (4, 35), and enhanced myogenic responses (9).

However, the exact mechanism(s) underlying this impaired muscle blood flow during exercise remains unclear.

In human studies, exercise has been shown to increase the release of vasodilatory prostaglandins, i.e., PGI\(_2\) and PGE\(_2\), suggesting that these factors may play an important role in the control of functional hyperemia (16, 39). Indeed, inhibition of prostaglandin formation has been shown to limit the increase in PGI\(_2\) and PGE\(_2\) in venous blood normally seen during exercise (39) as well as attenuating functional hyperemia (18, 39). PGI\(_2\) is synthesized from PGH\(_2\), a precursor prostaglandin and product of AA metabolism by cyclooxygenase, via prostacyclin synthase and is the principal prostaglandin synthesized by vascular endothelial cells (32, 33). In instances where PGIS activity is compromised, PGH\(_2\) accumulates and is shunted into formation of other prostaglandins as well as the vasoconstrictive product TXA\(_2\) (22, 32, 43). In the same studies measuring the increase in prostaglandin production during exercise (16, 17), TXA\(_2\) levels were shown to decrease during exercise. Thus, during exercise, the normal increase in PGI\(_2\) release coupled with a decrease in TXA\(_2\) synthesis would result in an overall vasodilatory response. Alterations in the normal metabolism of AA could lead to a decrease in the production of the vasodilator PGI\(_2\) and an increase in the production of the vasoconstrictor TXA\(_2\), thus leading to an overall impairment in vasodilation, which would potentially limit functional hyperemia. While previous studies from our lab and others have shown that the OZR exhibits impaired vasodilation in response to the PGI\(_2\) analog iloprost, it is unclear whether normal production of PGI\(_2\) is altered in this model of obesity (7, 42).

In the present study, we examined whether alterations in the normal AA metabolite profile may be a mechanism underlying the altered vasodilatory responses seen in the OZR.

To determine whether PGI\(_2\), PGE\(_2\), and TXA\(_2\) levels were altered in our model of obesity, we developed a protocol based on a similar one using cerebral arteries (24) to quantitate basal prostanoid production as well as stimulated prostanoid production in response to exogenous AA administration. Compared
with the other products measured (PGE2 and TXA2), PGI2 was
the predominant prostaglandin released by the femoral arteries
(Fig. 1). Basal levels of PGI2 were not significantly different
between the lean and obese animals; however, following incu-
bation with AA, PGI2 release from femoral arteries of obese
animals was significantly attenuated relative to arteries from
the lean animals. Interestingly, basal and stimulated TXA2
levels were not elevated in the obese as hypothesized (Fig. 2).
In fact, only arteries from the lean rats exhibited a significant
increase in TXA2 release following AA administration. PGE2,
while quantitatively produced in much lower quantities than
PGI2, exhibited a similar attenuation following AA adminis-
tration in the OZR arteries compared with the LZR arteries,
as well as no significant difference in basal levels (Fig. 3). Taken
Figure 1. Basal levels of PGI2 were not significantly different
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Taken together, these results suggest that AA metabolism is altered in
the OZR vasculature with an impairment in the synthesis pathways of the vasodilatory products PGE2 and PGI2, a
predominant regulator of functional hyperemia, and no con-
comitant increase in the production of the vasoconstrictive product TXA2. The PGE2 results indicate that other prostaglan-
din synthesis mechanisms may be impaired in our model. While human studies have shown that during acute and pro-
longed exercise PGE2 levels increase in the blood (3, 21, 39),
the role of this prostaglandin in skeletal muscle blood flow control is unclear. PGE2 has been studied more extensively in
vasculature beds, such as the renal circulation, and has been
shown to have four receptor subtypes, EP1–EP4. In the kidney
vasculature, EP2 and EP4 stimulation result in vasodilation,
whereas EP1 and EP3 stimulation result in vasoconstriction
(37). PGE2 may also play a role in other vasculature beds, such
as the cerebral circulation. Further work needs to be performed
to elucidate the role of PGE2 and the EP receptors in skeletal
muscle blood flow control.

Previous findings from our laboratory have suggested that the alterations in AA metabolism impair functional vasodila-
tion in the OZR through augmented TP receptor-mediated
vasoconstriction and attenuated IP receptor-mediated vasodi-
lation (42). However, it was unclear as to the identity of the AA metabolite responsible for the increase in TP receptor-mediated
vasoconstriction, since the TP receptor can be activated by
both TXA2 and its precursor PGH2 (29, 30). The lack of a
significant difference in basal and AA-stimulated TXA2 pro-
duction in the OZR supports the theory that the enhanced TP
receptor-mediated vasoconstriction in the OZR is due to an
accumulation of PGH2, resulting from a decrease in PGI2
synthesis and not an increase in TXA2 synthesis itself (12, 42).
This theory is also supported by studies in other animal models
and vascular beds in which enhanced vasoconstriction due to
AA administration has been shown to be attenuated following
TP receptor blockade but not following inhibition of TXA2
synthesis (26, 36, 40).

The finding that the OZR exhibits an attenuated PGI2 release
following AA administration (Fig. 1) suggests that in addition
to the previously mentioned decrease in sensitivity of the OZR
vasculature to IP receptor stimulation, this model of obesity
does in fact exhibit impaired PGI2 synthesis, which would
further limit vasodilation during exercise. Chronic elevations
in oxidative stress in the OZR may be the mechanism by which
this apparent decrease in PGI2 synthesis occurs. Frisbee (6)
has previously suggested that the impaired hypoxic vasodilation in
the OZR vasculature may be due to increased PGI2 degradation
by superoxide. However, the primary mechanism by which
elevations in oxidative stress may impair PGI2 release is likely
due to inhibition of PGIS (43). In cultured human endothelial
cells, high glucose concentrations have been shown to increase
superoxide formation, leading to an increase in peroxynitrite
formation. Peroxynitrite has been shown to cause inactivation
of the PGIS enzyme through nitration of tyrosine residues (2,
45, 46). This inactivation in PGIS results in an accumulation
of PGH2, which as previously mentioned, can cause vasoconstric-
tion through activation of the TP receptor. Thus, we investi-
gated whether the attenuated PGI2 release in our model could
be attributed to an increase in nitration of PGIS. We used an
immunoprecipitation protocol to isolate PGIS from thoracic
aorta homogenates from both lean and OZRs. Nitration of
PGIS was determined by incubating Western blots of immu-
noprecipitated PGIS with an antibody to nitrotyrosine (Fig. 6).
PGIS migrated as two sets of bands, an upper band near 50 kDa
and a lower band near 30 kDa. This banding pattern corre-
ponds to that seen in atherosclerotic vessels, with the multiple
bands possibly resulting from PGIS degradation products (44).
The lower band had an increased ratio of nitrated PGIS/total
PGIS in the OZR compared with the LZR, whereas the upper
bands showed lower levels of total nitration that was similar
between groups. This result suggests an increased nitration of
PGIS in the OZR, which corresponds to their already increased
oxidative stress levels. Thus, an increase in nitration of PGIS is
a potential mechanism explaining the impaired PGI2 synthesis
found in the in vitro studies.

In addition to measuring PGI2 and TXA2 levels in our model
of obesity, we examined whether the receptors for these AA
metabolites were altered in the OZR. In theory, a downregu-
lation of IP receptors and/or an upregulation of TP receptors
could potentially result in an altered functional hyperemic
response. To determine whether IP and TP receptor expression
were altered in the OZR rat microvasculature, we utilized an
immunofluorescence technique (15) in which VSMCs from
intramuscular gracilis arterioles were isolated and stained with
antibodies to either IP or TP receptors. Interestingly, we found
that the IP receptor immunofluorescence tended to be lower in
the OZR cells compared with those from the LZR (Fig. 4); how-
ever, this trend did not reach statistical significance. The
TP receptor immunofluorescence was similar between the two
groups (Fig. 5). These results suggest that neither an increase
in TP receptor expression nor a downregulation of IP receptors
in the OZR are likely to be primary mechanisms involved in
the altered vasodilatory response. The TP receptor results are
in contrast to those seen in carotid arteries in a diet-induced
mouse model, where TP receptor mRNA expression levels are
elevated relative to lean animals (36). Although TP receptors
do not appear to be upregulated in our model, an accumu-
lation of PGH2 due to diminished PGI2 synthesis could still poten-
tially result in increased TP-mediated vasoconstriction in the
OZR. We initially theorized that a decrease in IP receptor
levels could be a potential mechanism involved in the altered
functional hyperemia of the OZR. A decreased IP receptor
expression could potentially explain the diminished IP receptor
sensitivity to PGI2 in the OZR. Coupled with attenuation in
normal PGI2 synthesis, decreased IP receptor expression would
result in a diminished stimulus for vasodilation during exercise
in the OZR. However, our results at this time are inconclusive

as to whether IP receptor alterations play a role in the altered vasodilatory responses seen in the OZR. Further investigation is needed to explore the roles of prostanooid receptor expression in the altered vasodilation in the OZR. Other mechanisms play a role in the attenuated functional hyperemia in this model. We have recently found that impaired vasodilation in the OZR may be partially due to a decreased sensitivity of ATP-sensitive potassium channels (K\(_{\text{ATP}}\)), which are involved in the normal IP receptor-mediated vasodilation (13). Thus, upstream as well as downstream vasodilatory mechanisms responsible for AA-induced vasodilation are likely impaired in the OZR.

**Perspectives and Significance**

The results of the present study indicate that a decrease in PG12 synthesis in the OZR skeletal muscle vasculature may be a mechanism underlying the impaired vasodilation in response to exercise. In addition, the OZR exhibits an increase in nitration of tyrosine residues of PGIS, a mechanism which has been shown to decrease PG12 production. Furthermore, the OZR exhibited no elevation in TXA2 production and no up-regulation of the TP receptor. Thus, the altered AA metabolism underlying the impaired functional hyperemic response seen in the OZR is most likely due solely to alterations in the PG12 portion of the pathway, with little to no compounding impairment of the TXA2 response. The enhanced TP receptor-mediated vasoconstriction evident in the OZR is likely due to accumulation of PGH2 resulting from attenuation of normal PG12 synthesis and this metabolite’s ability to stimulate the TP receptor. Although many questions remain concerning the role of altered arachidonic metabolism in the attenuated functional hyperemia in obesity, it is anticipated that the present findings may play a role in the future development of treatment protocols that improve muscle blood flow during exercise in obese patients, as well as adding to the base of knowledge in this area of physiological research.

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