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Intermittent hypoxia induces NF-κB-dependent endothelial activation via adipocyte-derived mediators

Mary Y. K. Lee,1,2 Yan Wang,1 Judith C. W. Mak,1,2 and Mary S. M. Ip1,2

1Division of Respiratory Medicine, Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong; 2Research Centre of Heart, Brain, Hormone and Healthy Ageing, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong

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treatment of intermittent normoxia (IN) and IH were housed in two identical commercially designed chambers (OxyCycler model A84; Biospherix, Redfield, NY), which allowed for the application of IN or IH for 6 h daily, from 9:00 AM to 3:00 PM, for 6 wk. The oxygen (O_2) concentration in the chamber was continuously measured by an O_2 analyzer during the exposure. Air-exchange cycles of 2 min of 21% oxygen (room air) and 4 min of 10% oxygen were applied, resulting in alternating intervals of 90 s of 21% O_2, gradually lowering to a trough of 10% O_2 maintained for about 90 s toward the end of each cycle (20). This hypoxic profile was associated with arterial oxyhemoglobin saturations (SpO_2), which fluctuated between a peak of 90–95%, gradually decreasing to a nadir of 60–75%, for 10 times per hour, as measured using a pulse oximeter (MouseVentG500; Kent Scientific, Torrington, CT). The achieved SpO_2 pattern was similar to the reported levels in similar experimental settings (7, 13).

At the end of the 6-wk exposure, all rats were killed by an intraperitoneal overdose (100 mg/kg) of pentobarbitone. To determine whether or not chronic exposure to IH causes adipokine dysregulation in various depot-specific adipose tissues, subcutaneous, epididymal, and periaortic adipose tissues were collected and dissected in cold PBS for the determination of protein expression of proinflammatory biomarkers. Aortae with intact endothelium, which may be influenced by either circulating adipose tissue-derived factors or their perivascular adipose tissue were, snap frozen and stored at −70°C until detection of the protein expression of endothelial NO synthase [eNOS; phosphorylated (p) at ser1177], proinflammatory Erk/p38 MAPK signaling components, and inducible NOS (iNOS).

The animal care and experimental protocol for the present study were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATR 2371-11). The research works were performed in accordance with the American Physiological Society Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.

**IH Exposure and Cell Cultures**

**IH exposure.** To evaluate the direct impact of IH on adipocytes and endothelial EA.hy926 (EA) cells, these cells were subjected separately to the exposure of IH as described (21). Briefly, cells were maintained at 57% at 5% CO_2 in air. In the chambers (OxyCycler model A42; Biospherix), in which O_2 levels were alternated between 21% for 5 min and 1% for 10 min, for a total of 64 cycles (18 h). The percentage of O_2 in the cell culture medium fluctuated between 18% (normoxic phase) and a nadir of 4.5–5.0% (hypoxic phase) per cycle. Cells in the control group were maintained under normoxic condition (IN: 21% O_2 phase) and a nadir of 4.5–5.0% (hypoxic phase) per cycle. Cells in the control group were maintained under normoxic condition (IN: 21% O_2 phase) and a nadir of 4.5–5.0% (hypoxic phase) per cycle. Cells in the control group were maintained under normoxic condition (IN: 21% O_2 phase) and a nadir of 4.5–5.0% (hypoxic phase) per cycle.

**Adipogenic differentiation of preadipocytes.** Primary human preadipocytes-subcutaneous (HPA-s; passages 3-5) were obtained from ScienCell Research Laboratories (cat. no. 7220; Carlsbad, CA) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 20% heat-inactivated FBS ( Gibco, Grand Island, NY). After the culture medium reached 70–80% confluence in a T25 culture flask, adipogenic differentiation was induced by replacing the culture medium with a differentiation cocktail containing 10 μg/ml insulin (GIBCO), 500 μM isobutylmethylxanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), and 100 μM indomethacin (Sigma-Aldrich) in IMDM supplemented with 10% FBS for 3 days. This was followed by 2 days of maintenance in IMDM supplemented with 10% FBS and 10 μg/ml insulin. After 6 cycles of differentiation confirmed by lipid staining [Oil Red O staining (data not shown)], the cells were referred to as mature adipocytes (adipocytes). Before the experiment, adipocytes were cultured in fasting medium (3 ml IMDM containing 1% FBS and 10 μg/ml insulin) overnight before being subjected to IH. After IH exposure, cells were collected for protein extraction and evaluation of protein expressions for NADPH oxidase 4 (NOX4), Erk, p38, NF-kBp65, and iNOS. The adipocyte-derived conditioned media after IH exposure or under normoxic condition were collected, sterile filtered, and frozen at −70°C until future experiments. Levels of IL-6 and monocyte chemoattractant protein-1 (MCP-1) release in the conditioned media were measured.

**Endothelial cells.** Endothelial EA cells were originally obtained from American Type Culture Collection (cat. no. CRL-2922; ATCC, Manassas, VA) and were cultured in Dulbecco’s Modified Eagle Medium (DMEM GlutaMax) (GIBCO) supplemented with 10% FBS. Experiments were performed using cells at passages 6-11. Cells in six-well plates were cultured in fasting medium (1 ml DMEM containing 1% FBS) overnight before IH exposure. Treated cells were collected for protein extraction and evaluation of protein expressions for p-eNOS (ser1177), eNOS, p-Erk 1/2, Erk, p-p38, p38, and iNOS. Levels of IL-6 and MCP-1 release in the EA cell-derived conditioned media were measured.

**Influence of adipocyte-derived factors on endothelial cells.** To investigate the potential impact of adipocyte dysfunction on endothelial cells, conditioned media derived from cultured adipocytes under hypoxic (IH) and normoxic (IN) conditions were collected for treatment of EA cells in vitro. The conditioned media were diluted 1:1 with DMEM containing 1% FBS before treatment. Because all organs would be simultaneously vulnerable to IH exposure in vivo, all experiments were done on EA cultures under both IH or IN conditions. NF-κBp65 nuclear translocation and MCP-1 release were studied. To investigate the possible effect of IL-6 as an adipocyte-derived cytokone on endothelial cell activation, EA cells were pretreated with anti-human IL-6 antibodies (0.2 μg/ml; R&D Systems, Minneapolis, MN) or the respective control (IgG) before incubation with adipocyte-derived conditioned media. Furthermore, recombinant human IL-6 (rIL6; R&D Systems), at a concentration similar to that detected in IH-stimulated adipocyte-derived conditioned media, was added to the endothelial cells for evaluation of cGMP, MCP-1 release, and iNOS protein expression. The possible signaling mechanisms involved in endothelial cell activation by rIL-6, illustrating the effect of adipocyte-derived IL-6, were determined with the use of various selective inhibitors. Inhibitors of MAPK/ERK (PD98059, 10 μM; Merck Millipore, Darmstadt, Germany), p38 MAPK (SB203580, 10 μM; Cell Signaling Technology, Danvers, MA), and NF-κB inhibitor (SC514, 10 μM; Tocris, Avonmouth, Bristol, UK) were administered to the EA cells 45 min before exposure.

**Intracellular cGMP Measurement**

To investigate the influence of IH on NO bioavailability, intracellular cGMP ([cGMP]) levels were measured in duplicate by immunoassay (Biomedical Technologies, Stoughton, MA) in treated EA cells, according to the manufacturer’s instructions. Values were calculated as picomoles per milligram cellular protein and expressed as relative fold changes compared with the normoxic group.

**Assessment of Proinflammatory Mediator Expression in Adipose Tissues or Cell Cultures**

Tissue lysates from adipose tissues were prepared in ice-cold lysis buffer (Thermo Scientific, Waltham, MA) containing a cocktail of protease and phosphatase inhibitors (Thermo Scientific) and 1 mM phenylmethlysulfonyl fluoride (Thermo Scientific). The protein concentration was determined using the Bradford assay. Tissue lysates from subcutaneous, epididymal, and periaortic adipose tissues were then analyzed in duplicate for the protein expression of IL-6, MCP-1 (ELISA kits; BD Biosciences, San Jose, CA), and the content of angiotensin II (Ang II) (EIA kit; Cayman Chemical, Ann Arbor, MI) in periaortic adipose tissues from both IN- and IH-treated groups. For in vitro experiments, the protein release of IL-6 and MCP-1 in conditioned media derived from cultured adipocytes and EA cells were analyzed in duplicate (ELISA kits; BD Biosciences) according to the manufacturer’s instructions.
**Western Blotting**

Total protein lysates (30 μg) from adipose tissues, aortae, cultured adipocytes, and EA cells were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were incubated overnight with the antibodies against p-eNOS (ser1177) (BD-612393), eNOS (BD-610297), iNOS (1:1,000; BD-610432; BD Biosciences), p-p38 (CS-4511), p38 (CS-9212), p-Erk1/2 (CS-4370), NF-κBp65 (CS-4764) (1:1,000; Cell Signaling), NOX4 (SC-30141; 1:500), and Erk1 (SC-94; 1:1,000; Santa Cruz Biotechnology, Dallas, TX) at 4°C. This was followed by incubation with horseradish peroxidase-labeled secondary antibodies (Novus, Littleton, CO) before image detection by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). A control housekeeping protein, GAPDH, was used to demonstrate the levels of sample loading.

**Statistical Analysis**

All data are shown as means ± SE. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Comparisons between groups were performed using independent sample t-test where appropriate. Comparisons of more than two groups were carried out with one-way ANOVA followed by Tukey’s multiple-comparisons test. P values <0.05 were considered statistically significant differences.

**RESULTS**

**Basal and Stimulated Levels (IH Exposure) of Proinflammatory Mediators from Adipose Tissues in Rats**

The basal levels of IL-6 and MCP-1 protein expression in periaortic adipose tissue were significantly higher (>10-fold) compared with that of epididymal and subcutaneous adipose samples. Exposure of the animals to IH for 6 wk significantly enhanced the protein expression of IL-6 and MCP-1 in subcutaneous and periaortic but not in epididymal adipose tissues (Fig. 1, A and B). Periaortic adipose tissue displayed a significant upregulation of Ang II and iNOS after IH (Fig. 1, C and D). However, the circulating levels of IL-6 and Ang II were below detection limits of the assays used (data not shown).

**Endothelial NOS, Erk, p38, and iNOS in Rat Aorta After Exposure to IH**

The rat aorta, corresponding to the site of periaortic fat depot studied, was selected for evaluation of the vasculature after IH exposure. The protein expression of one of the phosphorylation sites, ser1177, which positively regulates eNOS activity, was studied and was significantly downregulated after IH exposure (Fig. 2A). In addition, IH also induced activation of the inflammatory signaling components, p-Erk 1/2 and p-p38 (Fig. 2, B and C) in aortic tissue compared with that of IN-treated rats. The protein expression of total iNOS in aorta was not different between IN- and IH-exposed groups (Fig. 2D).

**Activation of Cultured Adipocytes and Endothelial EA Cells After Exposure to IH**

Exposure to IH, compared with normoxia, increased the protein expression of NOX4 in cultured adipocytes significantly (Fig. 3A). At the same time, IH also augmented the protein expressions of p-Erk 1/2, p65, and iNOS in cultured adipocytes (Fig. 3, B–D). The p38 MAPK signaling component was not changed after IH (data not shown). The levels of IL-6 and MCP-1 secreted in conditioned media from cultured adipocytes were also augmented by IH exposure (Fig. 3, E and F).

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![Fig. 1. The protein expression of proinflammatory mediators in different depots of adipose tissues after intermittent hypoxia (IH). The protein expressions of IL-6 (A) and monocyte chemoattractant protein-1 (MCP-1) (B) in epididymal (n = 3), subcutaneous (n = 3), and periaortic (n = 5) depots are shown. Protein expression of angiotensin II [Ang II; n = 3 and 4 for IH and intermittent normoxia (IN) groups, respectively] (C) and inducible nitric oxide synthase (iNOS) (n = 4 and 5 for IH and IN groups, respectively) (D) in periaortic depot is shown. Values were normalized as milligrams protein for IL-6, MCP-1, and Ang II determination. Protein expression for iNOS was normalized to GAPDH. Error bars are means ± SE. *P < 0.05 compared with the corresponding control, IN.](http://ajpcell.physiology.org/ by 10.220.33.4 on October 14, 2017)
 Activation of EA cells by rIL-6 Treatment and/or IH

Recombinant IL-6 (rIL-6) instead of conditioned media derived from cultured adipocytes was used to treat EA cells. Treatment with rIL-6 (18 h) alone caused downregulation of the [cGMP], level in EA cells, consistent with that observed after IH exposure. Similarly, treatment of IH-exposed EA cells with rIL-6 caused significant further reduction in the [cGMP], level compared with IH alone. Nevertheless, there was no further reduction in the rIL-6 plus IH combination group compared with rIL-6 treatment alone (Fig. 6A). On the other hand, MCP-1 release from the cells after rIL-6 treatment or IH alone was significantly increased. Incubation with rIL-6 of IH-exposed EA cells caused additional induction of MCP-1 release compared with either rIL-6 or IH alone (Fig. 6B).

Role of Erk, p38, and NF-κB in rIL-6/IH-Induced Proinflammatory Response in EA Cells

The additive effect of rIL-6 and IH on elevation of MCP-1 release and iNOS protein expression was abolished after pre-incubation with PD98059 (MAPK/Erk inhibitor; 10 μM), SB203580 (p38 MAPK inhibitor; 10 μM), and SC514 (NF-κB/IκB kinase inhibitor; 10 μM) (Fig. 7, A and B). Under normoxic conditions, there were no significant inhibitory effects of the various inhibitors on rIL-6-induced MCP-1 release and iNOS protein expression, except for a significant reduction in iNOS protein expression in the PD98059-treated group.

DISCUSSION

The present study demonstrates that IH exposure of lean rats induced a proinflammatory phenotype in subcutaneous and periaortic adipose tissues as well as an impaired eNOS activity with inflammatory activation in the aorta. In support of a regulatory role of adipose tissues on the endothelium, IH-exposed cultured adipocytes showed activation of NOX4 and Erk/NF-κB signaling pathway, whereas its conditioned media, when added to endothelial cell cultures, evoked NF-κB activation and secretion of proinflammatory mediators from endothelial cells. Consequently, adipocyte-derived IL-6 was demonstrated to be a mediator in this interaction.
IH and Adipose Tissue Inflammation

The pathological impact of IH on adipose tissues has been suggested to contribute to functional alterations and upregulation of proinflammatory mediators, such as IL-6, MCP-1, TNF-α, and the oxidative product, malondialdehyde, in fat tissues of experimental animals (11, 30, 31). However, most earlier experiments focused on the evaluation of this impact on visceral fat, whereas there is limited information on other fat depots (11, 30, 31), probably because visceral fat may be more relevant for cardiometabolic diseases in humans (1, 30, 32).

The present study in lean rats shows that IH induces the production of proinflammatory components (IL-6, MCP-1) in the subcutaneous fat depot. Our in vitro findings on cultured subcutaneous adipocytes also demonstrate a similar augmented proinflammatory response as evidenced by the levels of IL-6, MCP-1, and iNOS, in line with previous in vitro studies (22, 37).

NOX4, a constitutive active enzyme responsible for the generation of reactive oxygen species, is expressed in various cell types, including adipocytes (19). A significant upregulation of NOX4 protein expression was seen in cultured adipocytes after IH exposure, consistent with an increased oxidative load in IH. In line with the upregulation of NOX4 expression as the possible upstream component activating Erk/NF-κB (19, 35), the present findings demonstrate a significant increase in Erk phosphorylation and NF-κBp65 protein expressions, as well as elevation of downstream proinflammatory mediators in IH-exposed adipocytes. It is proposed that this IH-induced adipose tissue inflammation may then exert a pathological effect on the vascular endothelium.

Another notable finding relates to the differential effect of IH on various adipose tissue depots in the in vivo rat model. In the context of adipose tissue biology, the most abundant depot in human body is subcutaneous white fat, which plays an initial role in the response to external stimuli such as a positive caloric balance (1, 16). When the subcutaneous fat can no longer respond to the stimulation and becomes dysfunctional, visceral fat may be affected (1, 16). Angiopoietin-like 4, one of the key players during atherogenesis, is present in subcutaneous adipose tissue, and its level correlated with the severity of
nocturnal hypoxemia in patients with sleep apnea (9), suggesting that subcutaneous adiposity may not be entirely an innocent bystander in cardiometabolic pathogenesis in the context of IH. The absence of increase in IL-6 and MCP-1 in visceral epididymal fat in the current findings may be related to the relatively mild IH profile used. Perivascular fat tissue, with its intimate anatomic relationship with the blood vessels, is recognized as an important visceral fat tissue (38) with a distinct physiological role in the regulation of vascular function and disease (6, 8). The present data demonstrate that, compared with other adipose tissues, including visceral epididymal and subcutaneous fat, the basal and IH-induced secretion of proinflammatory mediators (IL-6 and MCP-1) is higher (by 10-fold) in the periaortic depot. These findings are in agreement with other studies under different pathological conditions, including obesity, suggesting a regulatory role of perivascular adipose tissues in vascular inflammation (6, 8). In addition, the elevated protein expression of Ang II in periaortic fat depots after IH might play a modulatory role. Besides its important role in the regulation of arterial blood pressure, Ang II induces inflammation and vascular injury (2, 28) and colocalizes with IL-6 in human coronary atherosclerotic plaques (33).

**IH and Endothelial Inflammation**

Exposure to IH has been consistently reported to result in direct activation of the endothelium and/or accelerates atherosclerosis (9, 14, 21, 23, 24, 26, 41). In the present experiments, endothelial dysfunction was observed after exposure to IH both in vivo and in vitro, as evidenced by the reduced protein expression of p-eNOS (ser1177) (aorta and cultured EA cells) and the reduced cGMP level (cultured EA cells), which is another well-established marker for NO bioavailability (10). Activation of the MAPK signaling pathway (Erk and p38) in aortic tissue with intact endothelium in vivo indicates initiation of proatherogenic responses within the vasculature (15, 26, 36). The initiation of pathological events such as upregulation of NF-κB-dependent monocyte chemotactic activity attribut-
able to reduced NO bioavailability (42) may be anticipated, given the IH-induced decrease in p-eNOS and elevation of MCP-1. Despite an increase in protein expression of iNOS in cultured endothelial cells after IH, the level of iNOS expressed in aorta was comparable between IH and control groups, in contrast to that reported in a previous animal study (14), and may be due to differences in IH duration and severity.

Adipose Tissue-Endothelium Interactions

Several studies have reported the negative impact of IH on the endothelium, as discussed (9, 14, 21, 23, 24, 26, 41). However, there are limited data regarding the regulatory impact of adipose tissues on the endothelium in the context of IH. The acceleration of atherosclerotic lesion formation in athero-
sclerotic-prone ApoE/−/− mice after IH exposure can be improved after epididymal lpectomy (30). This observation suggests a possible contribution of adipose tissue dysfunction in IH-induced atherogenesis. In agreement, we found an elevation of NF-κBp65 nuclear translocation and MCP-1 release in endothelial cells after direct incubation with IH-exposed adipocyte-derived conditioned media. It has been reported previously that IL-6 participates in endothelial dysfunction and vascular pathogenesis via inflammatory activation of NF-κB signaling pathway (33, 34). The attenuation effect produced by neutralizing antibodies against IL-6 strongly supports a mediating role of IL-6 in the adipocyte-derived conditioned medium. In confirmation, the application of rIL-6 produced similar activation as IH-exposed adipocyte-derived conditioned media, which was inhibited after pretreatment with the selective inhibitors for Erk, p38 MAPK, and NF-κB, providing a mechanistic pathway for the interaction between adipocytes and endothelial cells.

Of note, the direct proinflammatory action of IH on endothelial cells was diminished in the presence of adipocyte-derived conditioned media. In IH-exposed endothelial cells, an additive increase in MCP-1 release was seen on treatment with rIL-6, but this was not observed when the cells were treated with IH-stimulated adipocyte-derived conditioned media. This suggested the possible presence of anti-inflammatory factors in adipocyte-derived conditioned media and could reflect a physiological response to mitigate the adverse impact of IH in vivo. The biological system is equipped with various homeostatic mechanisms, so it is likely that multiple mediators, pathogenic or protective, would be at play in IH-associated atherogenesis.

Limitations and Significance

Several caveats should be noted in the interpretation of the present findings. First, using the IH model as a surrogate for OSA carries inherent limitations. The literature on IH models
comprises diverse IH profiles, and this could result in different metabolic responses, making comparisons of data between studies difficult. However, this may be analogous to the possible human scenario of OSA, which encompasses a wide range of IH profiles, which may even vary from night to night within the same individual. There is further limitation in replicating the IH profile of the in vivo model for in vitro experiments. Nevertheless, our in vitro model can achieve swings in partial oxygen pressure in the medium for each IH cycle. Furthermore, IH is only one factor, albeit a prominent one, in OSA, which carries multiple pathophysiological factors, including hypercapnia, pressure swings, sympathetic activation, cerebral arousals, and sleep fragmentation. Second, in the present study, we have only used cultured subcutaneous adipocytes in vitro for further interrogation of adipose tissue–endothelium interactions. We have also assumed that adipocytes form the predominant cell type in adipose tissue in the lean rat and account for the major effects of such tissues. We did not exclude the contribution of activated macrophages (especially in the context of obesity) in adipose tissue inflammation. Finally, the present study selected IL-6 and MCP-1 as the potential adipocyte-derived candidates for initiation of endothelial dysfunction in the context of IH. Nevertheless, it is plausible that other adipose tissue-derived mediators may also be involved.

In summary, the present study demonstrates a pathological impact of IH on adipose tissue biology and the possible cross talk between adipocytes and endothelial cells (Fig. 8). Adipose tissue may exert its effect on the endothelium either from a distance through the blood circulation or from a perivascular depot through paracrine effects. It is conceivable that IH-induced adipocyte cellular dysfunction may contribute to a proatherogenic milieu and further promote inflammatory endothelial activation in the context of clinical conditions with intermittent hypoxia, such as sleep apnea.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.Y.L., J.C.M., and M.S.I. edited and revised manuscript; M.Y.L., and Y.W. prepared figures; M.Y.L. and J.C.M. drafted manuscript; M.Y.L. and Y.W., J.C.M., and M.S.I. interpreted results of experiments. Finally, the present study selected IL-6 and MCP-1 as the potential adipocyte-derived candidates for initiation of endothelial dysfunction in the context of IH. Nevertheless, it is plausible that other adipose tissue-derived mediators may also be involved.

In summary, the present study demonstrates a pathological impact of IH on adipose tissue biology and the possible cross talk between adipocytes and endothelial cells (Fig. 8). Adipose tissue may exert its effect on the endothelium either from a distance through the blood circulation or from a perivascular depot through paracrine effects. It is conceivable that IH-induced adipocyte cellular dysfunction may contribute to a proatherogenic milieu and further promote inflammatory endothelial activation in the context of clinical conditions with intermittent hypoxia, such as sleep apnea.

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Author contributions: M.Y.L., J.C.M., and M.S.I. conception and design of research; M.Y.L. and Y.W. performed experiments; M.Y.L., Y.W., and J.C.M. analyzed data; M.Y.L., J.C.M., and M.S.I. interpreted results of experiments; M.Y.L. and Y.W. prepared figures; M.Y.L. and J.C.M. drafted manuscript; M.Y.L., J.C.M., and M.S.I. edited and revised manuscript; M.Y.L., J.C.M., and M.S.I. approved final version of manuscript.

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