Signaling Components Involved in Leptin-Induced Amplification of the Atherosclerosis-Related Properties of Human Monocytes

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Leptin • Monocytes • Atherosclerosis • Cell adhesion • Cell migration • CD36 • Oxidized low-density lipoproteins

Abstract
Background/Aims: Leptin, a 16-kDa cytokine that is released mainly by the adipose tissue, is known to affect a wide assortment of processes, ranging from energy homeostasis to angiogenesis and the immune response. In the present study, the effect of leptin on atherosclerosis-related properties of human monocytes was investigated.
Methods: Monocytes were isolated from whole blood obtained from healthy donors who had normal body mass index values. Pharmacological inhibition of specific signaling proteins was implemented. Fluorescence spectrometry and immunofluorescence techniques, as well as ELISA methods, were utilized. Leptin dose response curves were determined for each type of experiment.
Results: Leptin (160 ng/ml) was found to augment monocyte adhesion to laminin-1 and its migration through this glycoprotein, which is one of the main components of the extracellular matrix. Additionally, leptin increased CD36-receptor surface expression, as well as moderately oxidized low-density lipoprotein (oxLDL 3 ) uptake levels.
Conclusion: Leptin amplifies the pro-atheromatic properties of human monocytes through a complex signaling net which involves the Na+/H+ exchanger isoform-1, the actin cytoskeleton, phosphoinositide 3-kinase, certain conventional isoforms of protein kinase C and NADPH oxidase.

Introduction
Leptin is a 16-kDa adipokine, a cytokine produced by the adipose tissue in response to excessive food intake. It is an anorexigenic hormone, a satiety indicator, that plays a part in regulating energy homeostasis in the body by concurrently lowering appetite and increasing energy expenditure [1]. The primary target of leptin is probably the hypothalamus, where it can affect appetite directly. However, Ob-Rb, the main functional leptin receptor, has been shown to be expressed in a remarkably wide array of cell types, not just in the hypothalamus. Today it is commonly accepted that leptin exerts a pleiotropic effect, partaking in the regulation of such diverse processes as angiogenesis and the immune response [2, 3].

Obesity often coincides with hyperleptinemia, the latter being a consequence of the former as obese individuals are often desensitized to leptin. Their leptin resistance is a result of inherited mutations that affect the correct expression of leptin or its receptor [4].
Human monocytes have been shown to express Ob-Rb and are thus affected by leptin [5]. This is important, considering the fact that monocytes are cells of the immune system that participate in crucial defensive processes such as the inflammatory response, phagocytosis of intruding organisms or particles and the respiratory burst. The latter two phenomena are known to be implicated in the appearance and progression of atherosclerosis and are both mostly associated with macrophages, the cells into which monocytes differentiate [6]. Several studies, in both mice and humans, have demonstrated that leptin can increase the risk of vascular thrombosis [7-9].

Atherosclerosis is one of the main causes of morbidity and mortality in the developed world. It is manifested as gradual arterial stenosis, which over time severely limits blood flow through the artery, thus depriving adjacent tissues of essential nutrients. The consequences of this vary from minor debilitating effects to death, depending on the site of the obstruction and the affected artery [10].

Arterial stenosis is generally observed at arterial locations where lesions have occurred and which constitute potential sites of inflammation. Among the inflammatory events triggered at these areas is the elevated production of chemokines such as monocyte chemotactic protein-1, whose concentration gradients guide leukocytes to the sites of inflammation [11]. Circulating monocytes attracted in this way adhere and migrate into the arterial wall. There they differentiate into macrophages, cells which can phagocytose oxidized low-density lipoproteins (oxLDLs) through scavenger receptors such as CD36. Due to their inability to degrade oxLDLs, macrophages accumulate oxLDLs, finally becoming foam cells, the fundamental ingredients of atheromatous plaques, which are principally responsible for arterial stenosis [12, 13].

It has been noted in monocytes that the leptin and insulin pathways overlap at certain points [14]; however, the mode of leptin action and the signaling pathways that are utilized, particularly in relation to atherosclerosis, have not yet been fully elucidated. The present study focuses on the investigation of the effects of leptin signaling in human monocytes in relation to certain atherosclerotic properties, namely monocyte adhesion to and migration through the extracellular matrix component laminin-1, CD36 scavenger receptor expression and oxLDL uptake. Pharmacological inhibition of selected proteins known for their involvement in major signaling pathways was implemented in order to deduce their effects on leptin signaling in relation to the aforementioned properties.

### Materials and Methods

#### Materials

Recombinant human leptin was sourced from R&D Systems (Minneapolis, Minn., USA). Ficoll-Paque Plus (1.077 g/ml) and Percoll (1.130 g/ml) were from Amersham Biosciences (Piscataway, N.J., USA). Anti-human CD14 R-phycocerythrin conjugated antibody, hexadecyltrimethylammonium bromide and dianisidine dihydrochloride tablets, diphenylethylenodionium chloride (DPI) and Nω-nitro-L-arginine methyl ester hydrochloride (1-NAME) were from Sigma (St. Louis, Mo., USA). Low-density lipoprotein (LDL) and bovine serum albumin were from AppliChem (Darmstadt, Germany). Anti-human CD36 FITC-conjugated antibody was from Serotec (Kidlington, UK). Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) was from Invitrogen (Carlsbad, Calif., USA). Cariporide was from Sanofi-Aventis (Paris, France). Iscove's Modified Dulbecco's Medium (IMDM), fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine were from Biochrom (Berlin, Germany). Cytochalasin D and wortmannin were from Fluka (Buchs, Switzerland). GF109203X and Gö6976 were from Alexis (Lausen, Switzerland). All other reagents were of analytical grades and were obtained from commercial sources.

#### Leptin and Inhibitors

The leptin concentration used was 160 ng/ml (10 nM), which is within the range used in other studies on leptin and which can be observed in patients suffering from hyperleptinemia, a direct result of severe obesity and its associated leptin resistance [5, 15]. Moreover, dose dependence measurements for each of our experiments showed that this concentration was more than adequate to permit the manifestation of the monocyte atherosclerosis-related properties we studied. Cariporide (20 nM) inhibits the Na+/H+ exchanger isoform-1 (NHE1). Cytochalasin D (2 μM) disrupts actin polymerization. Wortmannin (50 nM) inhibits phosphoinositide 3-kinase (PI3K). GF109203X (10 μM) inhibits all protein kinase C (PKC) isoforms. Gö6976 (500 nM) inhibits α and β isoforms of PKC (PKCα, PKCβII and PKCγII). DPI (10 μM) inhibits NADPH oxidase and nitric oxide synthase. 1-NAME (100 μM) inhibits solely nitric oxide synthase.

#### Monocyte Isolation

Monocytes were isolated from heparinized whole blood from healthy individuals, as described previously [16]. Informed consent was given by all blood donors. In brief, heparinized whole blood was diluted with phosphate buffered saline (PBS) 1X (1 mM EDTA, pH 7.2) and washed twice with PBS 1X. Heparinized whole blood was then diluted with complete IMDM and overlayered on 46% Percoll in 50-ml Falcon tubes. After centrifugation (550 g for 30 min, room temperature, no brake) the peripheral blood mononuclear cell (PBMC) layer was collected and put in new, clean 50-ml Falcon tubes. The PBMCs were washed 3 times with PBS 1X (1 mM EDTA, pH 7.2). They were then diluted with complete IMDM and overlayered on 46% Percoll in 50-ml Falcon tubes. After centrifugation (550 g for 30 min, room temperature, no brake) the monocyte layer was collected, diluted with PBS 1X (1 mM EDTA, pH 7.2) and washed twice with PBS 1X before being used in the experiments. Monocyte purity in the final samples was measured on a Beckman Coulter EPICS XL-MCL flow cytometer using the CD14 antibody and was found to be >85%.

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**References**

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Monocyte Adhesion Assay

Laminin-1 was diluted in PBS 1× (pH 7.2) to a final concentration of 37.5 μg/ml. 50-μl aliquots of the solution were placed in each of the wells on a 96-well polystyrene plate, and left uncovered to dry overnight at room temperature. 100 μl of monocytes (10⁶/ml) in complete IMDM were added to each well. Additionally, 2–4 Eppendorf tubes were also filled with 10⁶ cells to be used later as 100% myeloperoxidase (MPO) controls. The cells were then incubated with the appropriate inhibitors for 15 min at 37°C, followed by incubation with leptin for 30 min at 37°C. Non-adhered cells were removed by washing the plates with PBS 1× (pH 7.0) and monocyte binding was quantified using the myeloperoxidase assay as previously described [17]. In brief, the cells were lysed using 0.5% (w/v) hexadecyltrimethylammonium bromide in PBS (pH 6.0) for 30 min at 37°C. After lysis, 50 μl of 0.2 mg/ml dianisidinedihydrochloride in PBS (pH 6.0) containing 0.4 mM H₂O₂ were added to each well. After 15 min, the MPO activity of the lysate was measured spectrophotometrically at 405 nm, using an ELISA reader. The Eppendorf tubes were centrifuged, the supernatant was removed, and the sedimented cells were lysed, transferred to clean wells and myeloperoxidase activity was measured as described above. The number of bound cells was estimated by comparing the optical density of the test wells with the optical density of the reference wells.

Monocyte Migration Assay

Transmigration of monocytes through laminin-1-coated filters was performed as previously described [18]. In brief, filters of 6.5 mm diameter, 5-μm pore Transwell culture inserts (Costar, Cambridge, Mass., USA) were coated with 20-μg/ml laminin-1 and left to dry overnight at 4°C. Non-adhered molecules were removed, and the filters were then blocked with 150 μl of 0.5% bovine serum albumin by incubation for 1 h at 37°C. Monocytes (5 × 10⁵ cells/insert) were incubated in 250 μl of complete IMDM without FCS and the appropriate inhibitors were then added to the top chamber of the insert while 500 μl of IMDM containing 10% FCS was added to the lower chamber. The plates were incubated for 15 min at 37°C followed by incubation with leptin for 30 min at 37°C. After incubation, non-migrated cells were removed very carefully through the use of cotton tips immersed in PBS 1× (pH 7.0). The cells on the filters were fixed and stained with a Hemacolor staining kit (Merck) and left to dry for 10 min at room temperature. The complete IMDM was then removed from the lower chambers and 200 μl of 10% acetic acid was added to each one in order to remove the bound dye. After gentle shaking for 3–4 min, the contents of the lower chambers were transferred to wells on a 96-well plate and measured, as previously described, at 590 nm in an ELISA reader [19].

CD36 Receptor Measurement

Monocytes were incubated with the appropriate inhibitors for 15 min at 37°C, which was followed by incubation with leptin for 30 min at 37°C. The controls were incubated in parallel at 37°C. The CD36 receptor FITC-conjugated antibody was added to all samples, which was followed by incubation for 10 min at 37°C. Monocytes were then washed 3 times with PBS 1× (pH 7.0) and fluorescence was measured under continuous magnetic stirring at 20°C in a 3-ml quartz cuvette using a Shimadzu fluorescence thermostatic spectrophotometer, with the excitation and emission wavelengths set at 495 and 525 nm, respectively.

oxLDL Uptake

Preparation of Dil-oxLDLs. LDLs were oxidized and then labeled with Dil towards Dil-oxLDL as previously described [20, 21]. In order to produce oxLDLs, LDLs (0.2 mg/ml) were incubated with 5 μM CuSO₄ in PBS 1× for 3 h at 37°C. Similarly, LDLs (2 mg/ml) were incubated with 50 μM CuSO₄ in PBS 1× for 24 h at 37°C in order to produce oxLDLs. Both reactions were terminated with the addition of EDTA to a final concentration of 0.25 mM. Both types of oxLDLs were then incubated with Dil for 18 h at 37°C, at a ratio of 300 μg of Dil to 1 mg of oxLDLs. This was followed by ultracentrifugation at 39,000 rpm for 24 h at room temperature using a fixed angle rotor (Ti 50) and dialysis against PBS 1×. Finally, oxLDLs were sterilized by passing them through a 0.45-μm filter. The final concentrations of the obtained Dil-oxLDLs were quantified by the Bradford method.

Experimental Procedure. Monocytes were incubated with the appropriate inhibitors in FCS-deficient complete IMDM medium for 15 min at 37°C. Leptin was then added with Dil-oxLDL (100 ng/ml) and monocytes were incubated at 37°C for the desired time. After this, monocytes were washed 3 times with PBS 1× (pH 7.0) and oxLDL phagocytosis was quantified by the measurement of fluorescence under continuous magnetic stirring at 20°C in a 3-ml quartz cuvette using a Shimadzu fluorescence thermostatic spectrophotometer, with the excitation and emission wavelengths set at 520 and 578 nm, respectively.

Statistical Analysis

Values are expressed as arithmetic means ± standard deviations (SDs). Comparison between groups was conducted using the 2-tailed paired t test and the 1-way analysis of variance with the Student-Newman-Keuls test. p < 0.05 was used as the minimum accepted significance level. For statistical evaluation, the statistical software GraphPad InStat version 3.00 was used (GraphPad Software, San Diego, Calif., USA).

Results

Dose-Dependent Effect of Leptin

The leptin concentration we used (160 ng/ml) appeared capable of delivering an approximate peak effect for all the monocyte atherosclerosis-related properties we studied (fig. 1).

Adhesion to Laminin-1

The incubation of monocytes with 160-μg/ml leptin resulted in a 1.89-fold increase (p < 0.05) in adhesion to laminin-1 compared to the control population (fig. 2). The leptin sample value, in particular, corresponded to 78.2% of the MPO 100% (myeloperoxidase 100%, the expected value if all the cells had adhered to laminin-1) compared to just 41.5% for the control sample. All the inhibitors used, apart from l-NAME, attenuated this effect of leptin.
Migration through Laminin-1

Incubation of monocytes with 160-ng/ml leptin resulted in a 1.39-fold increase (p < 0.05) in migration through laminin-1 (fig. 3). The results obtained after incubation with the inhibitors indicate the involvement of NHE1, the actin cytoskeleton, PI3K and one or more of the \( \alpha \), \( \beta_1 \) and \( \beta_{11} \) isoforms of PKC in this leptin-induced mechanism.

CD36 Receptor Expression Measurement

Incubation of monocytes with 160-ng/ml leptin resulted in a 1.5-fold increase (p < 0.05) in CD36 receptor surface expression compared to the control monocyte population (fig. 4). Subsequent incubation with the available inhibitors indicated the involvement of NHE1, PI3K, NADPH oxidase and one or more of the \( \alpha \), \( \beta_1 \) and \( \beta_{11} \) isoforms of PKC in this leptin-induced mechanism.
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Forms of PKC in the leptin-mediated CD36 receptor-surface expression.

**oxLDL₃ Uptake**

Incubation of monocytes with 160-ng/ml leptin resulted in 1.54-fold and 1.64-fold increases (both p < 0.05) in oxLDL₃ uptake after 1 and 3 h, respectively, compared to the control population (fig. 5). Interestingly, incubation with 160-ng/ml leptin for 5 h did not result in a statistically significant increase in oxLDL₃ uptake. The addition of cariporide or wortmannin attenuated the effect of leptin after both 1 and 3 h (particularly after 1 h), thus indicating the involvement of NHE1 and PI3K. Additionally, NADPH oxidase appears to have a role, as its inhibition also attenuates leptin-mediated oxLDL₃ uptake after 1 and 3 h.

**Effect of Inhibitors on Control Samples**

We measured the effect of the inhibitors on the processes studied (cell adhesion, cell migration, CD36 surface expression and oxLDL₃ uptake), as well as on cell viability in the absence of leptin. At the concentrations
we used, the inhibitors in the absence of leptin did not affect in a statistically significant way either the processes we studied (table 1) or cell viability (data not shown).

Discussion

During early atherogenic events, monocytes pass through the arterial walls into areas where lesions exist. Once there, monocytes can cause damage to the endothelium by excessively producing reactive oxygen species, thus creating new lesions or enlarging pre-existing ones. The resulting inflammation leads to the formation of atheromatous plaques, of which leukocytes and monocytes in particular, in the form of foam cells, are fundamental components [22]. Adhesion to and migration through laminin-1 serve as good representations of the elemental procedures that must occur for monocytes to reach the area of inflammation. After interaction between monocytes and the extracellular matrix has been established, the monocytes migrate to the lesion area through a particularly complex procedure, guided by chemotactic stimuli produced by chemokines such as monocyte chemotactic protein-1 [24]. This procedure involves interactions between cell adhesion molecules on the surface of monocytes (mainly selectins and integrins) and their ligands on endothelial cells, as well as components of the extracellular matrix. Moreover, a previous study has shown that leptin can activate microvascular endothelial cells, thus promoting monocyte diapedesis and their differentiation into tissue-resident macrophages [25]. We found that leptin had a significant positive effect on monocyte migration through laminin-1. This effect is possibly mediated through an increased surface expression of the appropriate integrins, since interaction between integrin and laminin-1 in monocytes and other cells has been previously involved in the atherogenic process.

Fig. 4. CD36 receptor surface expression. Monocytes were pre-incubated for 15 min at 37°C with the inhibitors, leptin (160 ng/ml) was then added and the sample was incubated for a further 30 min at 37°C. The arithmetic means of at least 6 experiments are shown. Error bars indicate SD. The level of significance of the differences between the samples was calculated by ANOVA and Student-Newman-Keuls post-test. a p < 0.05 versus control. b p < 0.05 versus leptin. See Methods for inhibitor concentrations.
reported [26, 27]. Our pharmacological inhibition results imply that NHE1, the actin cytoskeleton, PI3K and the α and/or β1 and β11 isoforms of PKC take part in this leptin-induced mechanism. The activation of NHE1 and the polymerization of actin that are required during cellular adhesion and migration are tightly regulated by a mechanism that involves certain GTPases, such as Rho A [28, 29]. Another GTPase, Rac, is thought to be involved in a positive feedback loop with PI3K that results in the promotion of cellular migration [28, 30], something which is in accordance with our findings.

Because of the high concentrations of reactive oxygen species in and around areas of inflammation, LDLs become oxidized (oxLDLs). In particular, reactive oxygen species released by the NADPH oxidase of endothelial cells, smooth muscle cells and, possibly, monocytes which reside in the area, are thought to play a crucial role in this oxidation process [31]. Moreover, nitric oxide (NO) produced by the NO synthase of the surrounding cells can interact with molecular oxygen and superoxide radicals to produce reactive nitrogen species which also have the capacity to oxidize LDLs to oxLDLs [32, 33]. Monocytes
and macrophages phagocytose oxLDLs and become foam cells. They grow in size as they cannot metabolize or dispose of oxLDLs, which eventually results in cell death that further fuels the pro-atherosclerotic inflammatory process [22]. The internalization of oxLDLs is mediated primarily by CD36, a scavenger receptor known to be expressed by monocytes and macrophages [34, 35]. A consensus has not yet been reached as to whether CD36 function is pro-atherogenic or protective against atherosclerosis. From one perspective, CD36 has a valuable role by removing toxic oxLDLs from the inflammation area. This removal of oxLDLs, however, is what leads monocytes and macrophages to become foam cells [36]. In our experiments, leptin was found to up-regulate the surface expression of CD36 receptor in human monocytes. These findings are in agreement with a recent study involving cardiomyocytes [37]. Specific pharmacological inhibition showed that NHE1, PI3K, α and/or β1 and β11 PKC isoforms and NADPH oxidase take part in this leptin-induced signaling mechanism.

Based on these findings, we investigated directly the effect of leptin on oxLDL internalization, the primary cause of foam cell formation. Leptin was found to increase the uptake of moderately oxidized LDLs (oxLDL3) by monocytes after 1 and 3 h of incubation. However, leptin did not seem to affect the uptake of the highly oxidized form of LDLs (oxLDL3) at the time points measured (1, 3 and 5 h, data not shown). This finding is in accordance with a previous study in which CD36 appeared to be more active towards mildly oxidized lipoproteins and less active towards fully oxidized lipoproteins [38]. Based on our results, we suggest that increased oxLDL3 uptake by human monocytes is also due to leptin-induced CD36 surface expression. The increase in oxLDL3 uptake induced by leptin was diminished after inhibition of NHE1, PI3K, Ob-Rb and can then activate STAT3, a transcription factor, which in turn mediates the expression of a variety of genes. At the same time, JAK2 can activate PI3K via the activation of insulin receptor substrate-2 [39]. PI3K is known to activate Ca2+-dependent PKCs (such as the α and/or β1 and β11 PKC isoforms), which then translocate to near the plasma membrane where they can interact with NHE1 [40, 41, 42]. Through another pathway involving the Rho GTPases, PI3K exerts control over the actin cytoskeleton, which in turn is anchored to the plasma membrane through NHE1 and the ezrin, radixin, moesin proteins [29, 43]. Our results suggest that the pro-atherosclerotic process further fuels the pro-atherosclerotic inflammatory response.
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The role of reactive oxygen species produced by NADPH oxidase is difficult to deduce. Our group has recently shown that monocytes from healthy individuals attach to a higher degree to oxidized laminin-1 [44]. As a microenvironment event, this could explain the role of NADPH oxidase in cell adhesion. However, its role in CD36 expression and oxLDL uptake requires further research.

In conclusion, we have demonstrated that leptin guides human monocytes in a pro-atherogenic direction. In particular, leptin increases the level of interaction between human monocytes and the extracellular matrix by favoring adhesion to and migration through laminin-1, while at the same time enhancing their ability to internalize moderately oxidized oxLDLs. In summary, NHE1, the actin cytoskeleton, PI3K, certain PKC isoforms and NADPH oxidase appear to be involved in the signaling mechanisms that regulate the aforementioned monocyte properties. However, their exact positions in these signaling mechanisms are difficult to pinpoint. Further research is necessary in order to fully elucidate the mechanism through which leptin promotes monocyte pro-atherogenic properties.

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