Human Recombinant Leptin Shows Dose and Time-Dependent Release of Nitric Oxide from Endothelial Nitric Oxide Synthase in Endothelium While on Angiogenesis

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Abstract

Nitric Oxide (NO) modulates various assortments of the angiogenic process. The endogenous hormone leptin is able to induce different physiological process such as angiogenesis at low concentration because of its high receptor specific affinity. Various studies speculated leptin's ability to induce endothelium-dependent vascular relaxation by stimulating NO through different signaling pathways. So far, no studies have reported the dose and time dependent potential of human recombinant leptin on NO release. Hence, an attempt has been made to understand the optimal concentration and time of incubation of human recombinant leptin for the enzymatic release of NO from endothelial Nitric Oxide Synthase (eNOS). Leptin induced changes in the localization and phosphorylation pattern of eNOS in cultured endothelium under various concentrations and time of incubation is studied. The 5 Nanomolar concentration of human recombinant leptin within 6 minutes of incubation could induce significant levels of NO from the activated eNOS in cultured endothelium through plasma membrane localization and phosphorylation of eNOS. Our findings suggest that human recombinant leptin can modulate NO-dependent new therapeutic avenue for angiogenesis-related disorders such as wound healing if used within the active concentration and time of incubation.

Introduction

Leptin, a 16 kDa adipocyte-derived hormone, plays a significant role in regulating energy homeostasis [1]. It also acts as a pro-angiogenic molecule and initiates the process of angiogenesis through the Ob-R receptor present on vascular endothelial cells (ECs) [2]. In our previous experiments we speculated that the human recombinant leptin could induce concentration and time dependent angiogenic potential and able to enhance the mRNA transcription level of Vascular Endothelial Growth Factor A (VEGF A) via PI3K/Akt/mTOR/s6Kinase signaling pathway on chick embryo vascular bed [3, 4]. Leptin-induced NO releases from eNOS in ECs through the activation of various canonical signaling pathway is considered as one of the critical events in angiogenesis [5, 6]. However, no studies have been reported about the concentration and time dependent ability to human recombinant leptin on the enzymatic release of NO from eNOS in ECs. Hence, in the present study, we try to understand the concentration and time dependent capacity of human recombinant leptin on NO release from endothelial eNOS through evaluating various cellular mechanisms such as the subcellular localization, phosphorylation, and the interaction with Caveolin-1 as determinate rate factors. Since leptin exerts its biological impact through its specific receptor, it is highly advisable to find the optimum working concentration and time of incubation for the recombinant protein to exert its maximum angiogenic potential while considering for therapeutic applications.

Materials And Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) is purchased from PAN Biotech and Foetal Bovine Serum (FBS) from Invitrogen Life Technologies (Gaithersburg, MD). Human recombinant leptin, Bradykinin, Phenylmethylsulfonyl fluoride (PMSF), Picoll, 5Fluorouracil (5FU), 5-Chloromethylfluorescein diacetate (CMFDA), and all the primary antibodies are purchased from Abcam, USA. Propidium Iodide (PI), L-NAME, L-Arginine, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), recombinant VEGF were from Sigma Chemical Co (St. Louis, MO). L-NIO is purchased from Enzo life sciences and Wortmannin from Santa Cruz Biotechnologies. Collagen type-1 purchased from Pan Biotech GmbH Am Gewerbeperk. DAF- FM (4 amino-5-methylamino-2'7'difluorescein) and Calcium green1/AM were from Molecular probes, Eugene, Oregon, USA. Protease inhibitor tablets from Roche Diagnostics. All the primary antibodies are purchased from Abcam, and all the secondary antibodies and DAB systems are purchased from Bangalore Gene, India. All the other chemicals are at least of the reagent grade and are obtained commercially.

Cell lines

The T24 bladder carcinoma cell line formerly designated as ECV 304, stably transfected with eNOS-GFP constructs, is a kind gift from Dr. Vijay Shah, Mayo Clinic, Rochester, USA, and was used for immunofluorescence analysis. Another immortalized endothelial hybrid cell line EA.hy926 is a kind gift from Dr. C.J.S. Edgell, University of North Carolina, Chapel Hill. The cells are cultured in DMEM medium supplemented with 10 % FBS (v/v) and 1 % penicillin/streptomycin (w/v) and maintained at 37 °C in a humidified CO₂ incubator. Bovine aortic endothelial cells (BAECs) are isolated from the bovine aorta. The aorta is collected from a government-authorized slaughterhouse. BAECs are separated according to protocols described elsewhere [7]. The isolated cells are confirmed as endothelial cells by using the antibody against endothelial marker eNOS. Primary endothelial cells are used till passage 6.

Eggs

Fertilized White Leghorn chicken (*Gallus Gallus*) eggs weighing 50 ± gms are obtained from the Poultry research station, Nandanam, Chennai, Tamil Nadu, India. The eggs are incubated at 37–38°C, at a relative humidity of 70 to 80% using an egg incubator. All experiments are performed based on the Hens Egg Test- Chorioallantoic Membrane (HET-CAM) method [8, 9].

Scratch monolayer wound healing assay

Scratch wound healing assay is performed using EA.hy926 cells as described elsewhere [7]. In brief, a monolayer scratch is made on EA.hy926 cells and incubated with 0, 1, 5, 10, 25, and 50 nM concentrations of leptin for 6 hours. Images are recorded every 2 hours of interval. To confirm leptin's temporal and after wash effect after 6 hours, the wounded area is washed with PBS and continued incubation up to 10 hours without leptin. The rate of healing is calculated from the images. The experiment is repeated with 5 nM concentration of leptin, and the effect is compared with 3 mM concentration of 5- Fluorouracil. Throughout the healing experiments, the cell-free area of the wound is recorded using a Nikon Camera attached with a Bright field microscope at 4X magnification. It is

subsequently analyzed using Image J software to calculate the percentage and rate of wound healing from the initial and final wounded areas.

Endothelial ring formation assay

Ring formation assay is performed as described elsewhere [10]. In brief, EA.hy926 cells are incubated with 0, 1, 5, 10, 25, and 50 nM concentrations of leptin for 6 hours. Images are recorded every 2 hours of the interval using a Nikon Camera attached with a Bright Field Phase Contrast microscope at 20X magnification. The rings formed by two or more endothelial cells are considered ring structures and are counted from every field. The rate of endothelial ring formation every 2 hours is calculated. The endothelial ring structure's stability under leptin is confirmed from real-time image analysis and compared with the L-NIO (0.01 μ M) effect. After the incubation, each coverslip is mounted on a customized live-cell chamber on a microscope stage and is fixed after focusing on a single ring structure. The cells are monitored for 30 minutes continuously. Live-cell images are recorded at 0, 10, and 30 minutes of incubation at 20X magnification using a Nikon Camera attached with a Bright Field Phase Contrast microscope.

Measurement of Nitric Oxide

The level of NO released from ECs after leptin incubation is measured by employing three methods. **(A)** DAF-FM method – The experiment was performed using EA.hy926 cells, as mentioned earlier [7]. Cells are treated with L-NIO (0.1 μ M) for 30 minutes before the incubation with 0, 5, 10, and 25 nM concentrations of leptin for 4 hours. After PBS washing, the cells are treated with 1 μ M DAF-FM in DMEM along with 1 mM of L-Arginine for another 30 minutes. The supernatant of 100 μ l in volume is collected from each group. Using a Varian Cary eclipse UV-vis fluorescence spectrophotometer, the OD is measured at an excitation/emission of 495/515nm wavelength. **(B)** Griess assay – Griess assay is performed as mentioned earlier [11], repeating the same experimental procedure described above. After the incubation with L-NIO and Leptin, cells were treated with 2X HBS and 1mM L-Arginine for another 30 minutes. The supernatant of 100 μ l in volume is collected from each group and mixed with an equal volume of PBS (50 μ l) and Griess assay reagent A and B. After 10 minutes of incubation, the OD value at 540 nm is measured using a Varian Cary 4000 UV-Vis Spectrophotometer. The nitrite concentration is determined from a standard curve generated with Sodium Nitrate (NaNO₂). The early NO-releasing capacity of 5 and 10 nM concentration of leptin for 3, 6, and 240 minutes of incubation is measured repeating the same protocol mentioned above. A comparative study is carried out using a known inducer of NO such as VEGF (10 ng) and an inhibitor, Bradykinin (1 μ M). **(C)** NO measurement using NO electrode - Direct measurement of NO immediately after the release from ECs was performed using an Apollo 4000 Free radical analyzer (at 37°C, pH 7.4), an optically isolated multi-channel free radical analyzer with a NO selective membrane as described elsewhere [7]. BAEC cells are incubated with 0, 5, and 10 nM concentrations of Leptin for 6 and 12 minutes. After the incubation, the cells are incubated with 1 mM L-Arginine for another 30 minutes. The real-time acquisition of NO release is measured at 100 nA^o voltage at 37°C and is recorded through a single-board computer. During the 9th minutes of the experiment, 5

and 10 nM concentration of Leptin is added separately, and the entire set-up is allowed to run for 30 minutes and compared with the control value for the same incubation period.

Analysis of eNOS-GFP localization pattern using eNOS-GFP stably transfected endothelial cells

As mentioned earlier, the localization pattern of eNOS in eNOS - GFP stably transfected ECs is performed [7]. ECV-304 eNOS GFP tagged cells are incubated with 0, 5, and 10 nM concentrations of leptin for 1st and 4th hours separately. After fixing and permeabilizing, the cells are incubated with 2 µL of DAPI (stock- 1 mg/ml) for 10 minutes and are mounted with 70% glycerol. Images are recorded at 60X magnification using a DP71 color camera attached with an Olympus 1X71 Epifluorescence microscope at 515 nm wavelength. The same procedure is followed for 0, 5, and 10 nM concentrations of Leptin for 3, 6, 9, and 12 minutes. The cells are categorized into the plasma or perinuclear membrane brightness based on the localization eNOS.

Fluorescence imaging analysis of the intracellular calcium in endothelial cells using Calcium Green Probe

Intracellular calcium level is measured as described earlier [7]. EA.hy926 cells were incubated with 0, 5, and 10 nM concentrations of leptin for 6 minutes, followed by incubation with 5 µM Calcium green1/AM for 30 minutes. Additional incubation with PBS is given for another 15 minutes and after washing with PBS for 3 times to complete hydrolysis of any intact ester linkages in the intracellular calcium green 1/AM. Coverslips were loaded in a live cell chamber and placed onto the stage of an Olympus 1X71 Epifluorescence microscope. Fluorescence images of the cells are recorded using a DP71 camera attached to the microscope, and the fluorescence intensity of the cells is calculated using Adobe Photoshop version 6.

Immunofluorescence method

Immunofluorescence analysis is performed as described elsewhere to evaluate the phosphorylation pattern of eNOS and co-localization of eNOS and caveolin-1 [7]. ECV-304 eNOS GFP tagged cells are incubated with 0 and 5 nM concentrations of leptin for 6 and 9 minutes. Followed by fixing and permeabilization, the cells are blocked with 0.5% of BSA for 10 minutes. Overnight incubation is done with primary antibodies of phospho Ser-1177 and with Caveolin-1 rabbit polyclonal primary antibody (1:1000). TRITC labeled secondary antibody (1:2000) is used for the detection, and the nuclear staining is performed with DAPI. Images are recorded at 60X magnification using a DP71 color camera attached with an Olympus 1X71 Epifluorescence microscope with the required filter.

Western blot method

(A) Direct western blot - Direct western blot method is performed as described earlier to analyze the protein level expression of phospho Serine 1177 and eNOS [7]. ECV-304 eNOS GFP tagged cells are treated with 0 and 5 nM concentrations of leptin for 6 and 9 minutes. Following the protein separation and wet blotting, the membrane is probed for anti-eNOS (rabbit polyclonal, dilution of 1:1000), phospho

eNOS (Ser-117, dilution 1:1000), and detected using HRP labeled secondary Ab of 1:2000 dilutions. The blots are developed using TMB/H₂O₂ substrate. Images of the membrane are recorded using BIO-RAD (USA) system. **(B) Western blot analysis after ultracentrifugation** – This experiment is performed to identify the localization of Caveolin-1 and eNOS in endothelial cell subcellular fraction after leptin incubation. The procedure is adopted from the protocol described elsewhere [12]. In brief, ECV-304 eNOS GFP tagged confluent cells are incubated with 0 and 5 nM concentrations of Leptin for 6 minutes, and after the ultracentrifugation, all the 10 fractions are carefully collected into sterile vials. The western blot analysis for Caveolin-1 and eNOS is performed using rabbit polyclonal antibodies (1:1000 dilution) as described above.

Egg yolk angiogenesis assay

Egg yolk (area vasculosa) angiogenesis assay is performed as described below. In brief, 2 to 3 mL of albumin is withdrawn on the 3rd day of incubation, and a square window is opened. During the 4th day of incubation, sterile filter paper disks soaked with 0 and 5 nM leptin, L-NAME (nitro arginine methyl ester) (1 μ M) and leptin +L-NAME are then placed on the egg yolks and incubated for another 8 hours at 37°C in an egg incubator. Images are taken at 4X magnification using a Nikon Cool Pix camera (Olympus India Pvt Ltd, New Delhi, India) adapted to a stereomicroscope at 0, 4, and 8 hours incubation. Quantification of angiogenesis is performed using AngioQuant Toolbox, MATLAB 6.5 (R13) software. Histological analysis is performed as described elsewhere [8, 9].

Statistical analysis

All the experiments are performed in triplicate or more ($n \geq 3$) until otherwise specified. Data are presented as mean $\pm$ SEM. Data analyzed using Student t-test as appropriate. Data with p-values equal or less than $p < 0.001$ is selected as a criterion for showing a statistically significant difference.

Results

Human recombinant leptin induce concentration dependent EC migration, proliferation, and ring formation.

Scratch wound healing assay is performed to find the ability of human recombinant leptin on EC migration and proliferation. Figure 1.a represents the images of the scratch wound before and after the incubation under various concentrations of leptin. From the data, it is clear that 5 nM concentration of leptin repopulated the denuded area by enhancing ECs migration significantly up to 69% within the 6 hours of incubation ($p = 0.005$, Fig. 1.b). This impact is further confirmed by measuring the healing effect of leptin at the wounded area after the wash effect (Fig. 1.c). Interestingly, it is found that after the wash, the healing rate of 25 and 50 nM concentrations of leptin (studied higher concentrations) is increased significantly ($p < 0.001$). One possible mechanism for such effect is that, after the wash, the effect of higher concentration of leptin is diminished due to them, and hence, the migratory and proliferation impact on ECs is strengthened and has become equal to the effect of leptin at a lower concentration. To

determine the migratory and proliferative potential of ECs under human recombinant leptin, we performed a scratch wound healing assay in the presence of an anti-proliferative agent, namely 5-Fluorouracil (5-FU) (Fig. 1.d). The result indicates that the healing effect of 5 nM leptin is reduced together with 5-FU (57%) (68%, $p = < 0.001$). This observation suggests that human recombinant leptin shows concentration-dependent wound healing potential by accelerating ECs migration and proliferation.

The human recombinant leptin's ability to form stable endothelial ring-like structures is analyzed as a quantitative method to prove its potential as a pro-angiogenic molecule. Endothelial ring structures are the fundamental units of capillary tubes and are essential for angiogenesis. Hence, we also analyzed leptin potential to form stable ring-like structures at the early incubation period by recording the live images of ECs ring-like structures at 0, 10, and 30 minutes of incubation. Among studied concentrations, 5 nM leptin shows a significantly higher ring-like structure formation capacity rate within the 4th hour of incubation ($p = < 0.001$, Fig. 1.e). The effect of leptin is compared with L-NIO, which is known for its inhibitory action on the endothelial ring-like structure formation (Fig. 1.f). Images indicate that ECs incubated with 5 nM leptin can form a stable ring-like structure within 10 minutes of incubation and be noted with strong podia attachment between the ECs and sustain the ability for a more extended period (even at 30 minutes of incubation). On the other hand, ECs treated with L-NIO show fewer podia attachments with weak endothelial ring-like structures throughout the incubation period. Interestingly, it is noted that under leptin and L-NIO incubation, ECs show immediate distraction on their ring-like structures because of L-NIO impact within 10 minutes of incubation. But the same cells can regain their ability to form stable ring-like structures throughout the remaining incubation period and suggest leptin impact.

Human recombinant leptin induces concentration and time-dependent NO-release from ECs.

The amount of NO released from ECs under various concentrations of human recombinant leptin is measured using DAF-FM and Griess assay methods and compared with the L-NIO (a non-selective inhibitor of NOS) effect. Figure 2.a and 2.b indicates that the 5 nM leptin concentration induces a significant level of the enzymatic release of NO from ECs than other studied concentrations ($p = < 0.001$). But, in the presence of L-NIO, leptin's NO-releasing ability has been reduced irrespective of its concentration. This result directly supports leptin's ability to activate eNOS in ECs, which is considered an important event for the enzymatic release of NO from ECs. The data indicate that both 5 and 10 nM concentrations of leptin can release a significant NO level from ECs. Further, we compared the NO-releasing potential of both 5 and 10 nM leptin with other known strong inducers of NO release, such as VEGF and Bradykinin. Figure 2.c indicates that both leptin concentrations show a significant increase in the level of NO release with comparatively more effect under 5 nM concentration ($p = < 0.001$), and the impact is almost equal to that of VEGF.

Griess assay is performed to measure the NO-releasing ability of 5 and 10 nM concentrations of leptin at 3, 6, and 240 minutes of incubation to determine the earlier optimum incubation period for the higher level of NO release from ECs. Interestingly, it is found that both 5 and 10 nM leptin-induced an elevated level of NO release at 3 and 6 minutes of incubation ($p = < 0.001$) (Fig. 2.d). Among the analyzed

concentrations, 5 nM leptin shows the maximum ability to release a higher level of NO enzymatically at 6 minutes of incubation. Thus, the result implicates that the 5 nM concentration of leptin can release a significant level of NO from ECs within 6 minutes of incubation. Reconfirmation of the observation is carried out using an ultrasensitive NO electrode, which conformed a higher peak in the level of NO release under 5 nM concentration of leptin at 6 of incubation (Fig. 2.e).

Human recombinant leptin regulates the localization pattern of eNOS at the subcellular level under Nanomolar concentration.

Human recombinant leptin's ability to regulate the localization of eNOS within ECs is analyzed under 5 and 10 nM concentrations using eNOS GFP tagged ECs at different incubation periods. Figure 3.a shows that during the 1st and the 4th hour of incubation, cells incubated with 5 nM concentration of leptin show more localization of eNOS at the plasma membrane. In contrast, the control cells are noticed with more perinuclear membrane localization of eNOS. The cells were categorized into the plasma membrane and the perinuclear membrane bright cells based on the localization of eNOS. Figure 3.b indicates that the pool of ECs incubated with 5 nM concentration of leptin show 68% of more plasma membrane bright cells during the 1st hour of incubation as an indication of more localization of eNOS at the plasma membrane upon leptin incubation. During the 4th hour, the pool of ECs shows 54% of more plasma membrane bright cells under 5 nM concentration of leptin and is significantly higher than 10 nM concentration effect ($p = < 0.001$). The result indicates that within one hour of incubation, 5 nM concentration of human recombinant leptin trans- localize eNOS from the perinuclear membrane to the EC's plasma membrane region. We experimented on various time points such as 3, 6, 9, and 12 minutes to clarify the above observation. Figure 3.c shows that the cells incubated with a 5 nM concentration of leptin induce more eNOS localization at the plasma membrane region at every time point of incubation significantly with noticeable impact early at the 6th minute of incubation ($p = < 0.001$, Fig. 3.d). Results concluded that 5 nM concentration of human recombinant leptin regulates the subcellular trans-localization of eNOS from the perinuclear membrane to the plasma membrane region in ECs within 6 minutes of incubation. This possibility further enhances the enzymatic release of NO from eNOS.

Human recombinant leptin stimulates ECs through the modification of intracellular calcium levels at Nanomolar concentration.

In ECs, activation of eNOS also determined by the level of calcium. Stimulation of ECs through the mobilization of intracellular calcium favors the activation of eNOS. Further, the activated eNOS could detach from the Caveolin-1 and can enzymatically release NO. Hence, the intracellular calcium level in ECs is measured under recombinant leptin incubation as a parameter to find out the activation pool of eNOS enzyme for the sufficient level release of NO. Figure 4.a shows the representative images for the calcium intensity in ECs under 5 and 10 nM concentrations of leptin within 6 minutes of incubation. The average intensity of calcium per cell is calculated, and a graph plotted. It is observed that the cells incubated with 5 nM leptin show more calcium intensity significantly than those incubated with 10 nM concentration ($p = 0.05$, Fig. 4.b). Thus, the finding strongly suggests that the human recombinant leptin

can stimulate ECs through the mobilization of intracellular calcium, favoring the enzymatic release of NO from activated eNOS.

Human recombinant leptin-induces eNOS activation via the phosphorylation of eNOS at Serine 1177 residue at Nanomolar concentration.

We analyzed the impact of human recombinant leptin on the phosphorylation of eNOS in favor of the enzymatic release of NO from ECs. Co-expression of eNOS and phospho eNOS (serine 1177 residue) is analyzed by immunofluorescence and protein blot methods. Figure 5.a represents the images of co-localization of eNOS and phospho eNOS at Serine 1177 for 6 and 9 minutes of incubation. Cells treated with 5 nM leptin show more phospho eNOS (Serine 1177) protein at the plasma membrane region, which indicated the active pool eNOS through its phosphorylation. Measurement of protein level also supports the same finding (Fig. 5.b). It is noted that 5 nM concentration of leptin shows a significantly higher protein level of expression for phospho eNOS at the plasma membrane zone within 6 minutes of incubation ($p < 0.001$, Fig. 5.c). Thus, it can conclude that the leptin-induced eNOS activation depends on the phosphorylation of eNOS at serine 1177 residue, which is considered a surrogate marker for eNOS activation in ECs.

Human recombinant leptin mediates the interaction of eNOS with Caveolin-1 at the plasma membrane zone at Nanomolar concentration.

Co-expression of eNOS with Caveolin-1 in ECs under human recombinant leptin is examined as a tool to identify the activation of eNOS. Figure 6.a represents the images of co-expression of eNOS with Caveolin-1 under 5 nM concentration of leptin in ECs for 6 minutes of incubation. Cells incubated with 5 nM leptin concentration show the co-expression of eNOS and Caveolin-1 at the plasma membrane region within 6 minutes of incubation. The finding is further supported by separating cellular compartments by the western blot method after the ultracentrifugation process. This finding enables finding the specific compartment that shows more co-expression of eNOS and Caveolin-1 (Fig. 6.b). Fragments 1, 2, and 3 represent the plasma fractions, 4, 5, and 6 represent the Golgi fractions, and 7, 8, 9, and 10 represent the nuclear fractions from ECs upon 5 nM concentration leptin incubation. The result shows that the co-expression of Caveolin-1 and eNOS is higher at the plasma membrane fraction under 5 nM concentration of leptin within 6 minutes of incubation. Thus, the co-expression pattern reveals that the interaction of eNOS with Caveolin-1 will play a significant role in human recombinant leptin-induced eNOS activation and the enzymatic release of NO from ECs through regulating its function at the plasma membrane region. This mechanism further supports the events of the easier availability of NO for angiogenic processes.

The angiogenic ability of human recombinant leptin enhanced through NO

The experiment is performed to evaluate the angiogenic effect of human recombinant leptin in an *in vivo* condition. The investigation is performed on the highly delicate vascular membrane of CAM at the beginning stage of early vascularization. Figure 7.a indicates that the area vasculosa incubated with a 5

nM leptin concentration is noticed with more vascular density at the incubated area. On the other hand, the impact is found to be diminished in the presence of L-NAME. Quantification of vessel growth in terms of tubule length and size (Fig. 7.b) indicates that leptin promotes vessel growth significantly ($p = < 0.05$). Histological evaluation (Fig. 7.c) suggests that the area vasculosa incubated with leptin shows many positive angiogenic responses such as bulged sinus terminalis and a tightly packed vascular mesodermal layer between the blood vessels. It also indicates the presence of large blood vessels with lumen filled with numerous nucleated RBCs. In comparison, the area vasculosa incubated only with L-NAME shows a loosely packed thin layer of ectoderm with no sinus terminalis. Control membrane shows a narrow sinus terminalis and small blood vessels with tightly packed vascular ectodermal layers and different extracellular spaces between the mesodermal layers. The data support human recombinant leptin's ability to mediate angiogenesis through NO under Nanomolar concentration.

Discussion

Various findings prompted that human recombinant leptin may serve as a regulatory factor for angiogenesis [3, 4]. NO plays a critical role in angiogenesis and enhances multiple processes in ECs such as cell survival, proliferation, migration [13, 14]. *In vitro* studies under many pathological conditions indicated that leptin could evoke PI3k independent Akt-eNOS phosphorylation pathway to induce NO release [15, 6]. Our *in vitro* experiments concluded that the Nanomolar concentration of human recombinant leptin evokes an increase in NO release from eNOS in ECs. The effect is comparatively equal to various NO agonists such as VEGF and Bradykinin [16, 17]. The significant finding of our study is that human recombinant leptin at Nanomolar concentration (5 nM) accelerates the release of NO from ECs within a shorter incubation period (6 minutes) to favor angiogenesis.

It has been reported that the optimal coupling of extracellular stimulation of NO within ECs depends on the subcellular localization of the eNOS [18]. Our data gives an insight into the interaction of leptin with eNOS in ECs. We found that human recombinant leptin at its Nanomolar concentration trans-locates eNOS from the perinuclear membrane to the plasma membrane and operates the procedure within shorter incubation period. Thus, this trans-localization pattern of eNOS under Nanomolar concentration of leptin within shorter incubation period as a significant stimulation point of the enzymatic release of NO from activated eNOS in ECs. The phosphorylation pattern of eNOS in various amino acid moieties plays a vital role in regulating the catalytic activity of eNOS. It is well known that the phosphorylation of eNOS at Serine 1177 could lead to enhanced activation of eNOS and become a critical regulation of NO release from ECs [7]. Our experiments notion obtained from the co-localization and phosphorylation of eNOS at serine 1177 moiety indicated that the human recombinant leptin could enhance the catalytic activity of eNOS *via* the phosphorylation of eNOS at serine 1177 residue. This phosphorylation mechanism further accelerates the significant level of NO releases from eNOS in ECs. Thus, the present study hints that the human recombinant leptin activates eNOS by modulating both the subcellular localization and the phosphorylation pattern under lower concentration and within minimal incubation time.

Intracellular calcium dynamics in ECs is a critical determinant factor for eNOS activation, which binds to the canonical CaM-binding domain in eNOS to accelerate its catalytic functions via NO [19]. Cellular trafficking of eNOS within ECs is also considered a key player in releasing NO from activated eNOS [18]. In our research work, we found that under human recombinant leptin the presence of both eNOS and Caveolin-1 is more at the plasma membrane region than at the nuclear membrane. This data confirms the Nanomolar concentration of human recombinant leptin's ability to translocate Caveolin-1 and eNOS from the Golgi apparatus (cytosol) to the plasma membrane region in ECs within a shorter incubation period. Our data also showed that upon human recombinant leptin incubation, intracellular calcium mobilization is increased in ECs. This hike in the mobilization, in turn, may help to release NO from activated eNOS under human recombinant leptin. Hence, the present study emphasizes that the localization, phosphorylation at Serine 1177 residue, and the physical association with Caveolin-1 at the plasma membrane along with the intracellular calcium mobilization reflect an active pool of eNOS enzyme under human recombinant leptin in ECs. Further the CAN assay reveals that the 5 nM concentration of human recombinant leptin could promote NO-dependent angiogenesis and is in accordance with the earlier report which support the concentration dependent angiogenic impact of human recombinant leptin on CAM vascular bed [3].

Conclusion

Overall, our data confirm that the Nanomolar concentration of human recombinant leptin accelerates the significant level of NO release from eNOS in ECs within a shorter incubation period. The mechanism of action is regulated through eNOS subcellular trafficking, phosphorylation at Serine 1177 in ECs and is further supported by interaction of eNOS with Caveolin-1 and is modulated in the presence of intracellular calcium level. Altogether, the present study offers a mechanism that recruits leptin-eNOS-phospho eNOS axis which could favor the NO-mediated angiogenic process. The findings also highlighted the beneficial therapeutic role of human recombinant leptin through establishing leptin-eNOS-NO axis-dependent angiogenic potential under Nanomolar concentration within shorter incubation period. This finding may open up a new avenue for the therapeutic application of human recombinant leptin under wound healing milieu.

Declarations

Funding

Not applicable

Conflicts

The authors declare no conflicts of interest.

Availability of data material

All data generated or analysed during this study are included in this published article.

Code availability

Not applicable

Author's contribution

Conceptualization, Methodology and Formal analysis and investigation – Reji Manjunathan (RM) and Swaraj Sinha (SS). Formal analysis and investigation - Akila Swaminathan (AS) and Dharanibalan Kasiviswanathan (DK). Conceptualization and suspension - Malathi Ragunathan (MR) and Suvro Chatterjee (SC).

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Not applicable

Statement of Ethics

The research work followed ICCVAM-Recommended Test Method Protocol: Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method originally published as Appendix B3 of "ICCVAM Test Method Evaluation Report": Published 2010. Available at: <http://iccvam.niehs.nih.gov/methods/ocutox/MildMod-TMER.htm>.

References

1. Brennan AM, Mantzoros CS (2006) Drug Insight: the role of leptin in human physiology and pathophysiology—emerging clinical applications. *Nat Clin Pract Endocrinol Metab* 2(6):318–327. doi:10.1038/ncpendmet0196
2. Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD et al (1997) Crystal structure of the obese protein leptin-E100. *Nature* 387(6629):206-9. doi: 10.1038/387206a0. PMID: 9144295
3. Manjunathan R, Ragunathan M (2015) In ovo administration of human recombinant leptin shows dose dependent angiogenic effect on chicken chorioallantoic membrane. *Biol Res* 10(1):29. doi:10.1186/s40659-015-0021-z. 48) .
4. Manjunathan R, Devarajan N, Ragunathan M (2021) Possible Mechanism of Human Recombinant Leptin-Induced VEGF A Synthesis via PI3K/Akt/mTOR/S6 Kinase Signaling Pathway while Inducing Angiogenesis: An Analysis Using Chicken Chorioallantoic Membrane Model. *J Vasc Res* 24:1–18. doi:10.1159/000516498
5. Goetze S, Bungenstock A, Czupalla C, Eilers F, Stawowy P, Kintscher U, Spencer-Hänsch C, Graf K, Nürnberg B, Law RE, Fleck E, Gräfe M (2002) Leptin induces endothelial cell migration through Akt,

which is inhibited by PPARgamma-ligands. *Hypertension* 40(5):748–754.

doi:10.1161/01.hyp.0000035522.63647.d3

6. Vecchione C, Maffei A, Colella S, Aretini A, Poulet R, Frati G, Gentile MT, Fratta L, Trimarco V, Trimarco B, Lembo G (2002) Leptin effect on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway. *Diabetes* 51(1):168–173. doi:10.2337/diabetes.51.1.168
7. Majumder S, Rajaram M, Muley A, Reddy HS, Tamilarasan KP, Kolluru GK, Sinha S, Siamwala JH, Gupta R, Ilavarasan R, Venkataraman S, Sivakumar KC, Anishetty S, Kumar PG, Chatterjee S (2009) Thalidomide attenuates nitric oxide-driven angiogenesis by interacting with soluble guanylyl cyclase. *Br J Pharmacol* 158(7):1720–1734. doi:10.1111/j.1476-5381.2009.00446.x
8. Manjunathan R, Ragunathan M (2015) Chicken chorioallantoic membrane as a reliable model to evaluate osteosarcoma-an experimental approach using SaOS2 cell line. *Biol Proced Online* 6:17:10. doi:10.1186/s12575-015-0022-x
9. Rema RB, Rajendran K, Ragunathan M (2012) Angiogenic efficacy of Heparin on chick chorioallantoic membrane. *Vasc Cell* 18(1):8. doi:10.1186/2045-824X-4-8. 4) .
10. Sinha S, Sridhara SR, Srinivasan S, Muley A, Majumder S, Kuppusamy M, Gupta R, Chatterjee S (2011) NO (nitric oxide): the ring master. *Eur J Cell Biol* 90(1):58–71. doi:10.1016/j.ejcb.2010.07.005
11. Nims RW, Cook JC, Krishna MC, Christodoulou D, Poore CM, Miles AM, Grisham MB, Wink DA (1996) Colorimetric assays for nitric oxide and nitrogen oxide species formed from nitric oxide stock solutions and donor compounds. *Methods Enzymol* 268:93–105. doi:10.1016/s0076-6879(96)68012-4
12. Chatterjee S, Cao S, Peterson TE, Simari RD, Shah V (2003) Inhibition of GTP-dependent vesicle trafficking impairs internalization of plasmalemmal eNOS and cellular nitric oxide production. *J Cell Sci* 116(Pt 17):3645-55. doi: 10.1242/jcs.00664
13. van der Zee R, Murohara T, Luo Z, Zollmann F, Passeri J, Lekutat C, Isner JM (1997) Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 18;95(4):1030-7. doi: 10.1161/01.cir.95.4.1030
14. Beltowski J, Wójcicka G, Borkowska E (2002) Human leptin stimulates systemic nitric oxide production in the rat. *Obes Res* 10(9):939–946. doi:10.1038/oby.2002.128
15. Graier WF, Myers PR, Rubin LJ, Adams HR, Parker JL (1994) Escherichia coli endotoxin inhibits agonist-mediated cytosolic Ca²⁺ mobilization and nitric oxide biosynthesis in cultured endothelial cells. *Circ Res* 75(4):659–668. doi:10.1161/01.res.75.4.659
16. Morbidelli L, Parenti A, Giovannelli L, Granger HJ, Ledda F, Ziche M (1998) B1 receptor involvement in the effect of bradykinin on venular endothelial cell proliferation and potentiation of FGF-2 effects. *Br J Pharmacol* 124(6):1286–1292. doi:10.1038/sj.bjp.0701943
17. Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM (2004) Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest* 113(4):516–527. doi:10.1172/JCI18420

18. Sowa G, Liu J, Papapetropoulos A, Rex-Haffner M, Hughes TE, Sessa WC (1999) Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J Biol Chem* 274(32):22524–22531. doi:10.1074/jbc.274.32.22524. 274) .
19. Navarro A, Anand-Apte B, Parat MO (2004) A role for caveolae in cell migration. *FASEB J* 18(15):1801–1811. doi:10.1096/fj.04-2516rev

Figures

Figure 1

The Nanomolar concentration of human recombinant leptin enhances ECs migration and proliferation. (a). Monolayer wound healing assay - Representative images of monolayer scratch wound made on EA.hy926 cells. (n=3, bar is 10 μ M). (b). Percentage of wound healing - 5 nM leptin concentration shows a significantly higher healing percentage (69%). (n=6, mean $\pm$ SEM, [#]p=0.005 and ^{*}p<0.001 compared to control). (c). Rate of healing after the wash - The potential healing effect of leptin at Nanomolar

concentration is analyzed as before and after wash effect. After the wash, the healing rate significantly increased under 25 and 50 nM concentrations of leptin at 8th and 10th hour of incubation. (n=6, mean $\pm$ SEM, *p<0.001 compared to control). (d). Comparison of leptin's wound healing potential with 5FU - After creating a monolayer scratch wound area on EA.hy926 confluent cells, the wounded area is incubated with a 5 nM concentration of leptin and 3 mM 5FU for 6 hours. The healing percentage of leptin alone incubation is 68%, which indicates enhanced EC migration and proliferation. The impact is reduced when the cells are incubated with leptin along with 5FU (57%). (n=6, mean $\pm$ SEM, *p<0.001 compared to control). (e). Rate of ring-like structure formation – Among studied concentrations, 5 nM concentration of leptin at the 4th hour of incubation shows a significantly higher ring-like structure formation rate. (n=6, mean $\pm$ SEM, *p<0.001 compared to control). (f). Leptin shows stable ring-like structures – The ability of the 5 nM concentration of leptin to form a stable ring structure is analyzed and compared with that of L-NIO. Upon 5 nM concentration of leptin incubation ECs show stable ring-like structures (arrow) immediately within 10 minutes. Whereas ECs incubated with L-NIO show maximum damage at the protrusion of ring-like structure (arrow). While in the combination, the ECs retained their capacity to form a stable ring-like structure after 10 minutes of incubation because of leptin impact. (n=6, bar is 10 μ m).

Figure 2

Human recombinant leptin shows dose and time-dependent eNOS activation and NO release. (a). DAF-FM and (b) Griess assay – EA.hy926 cells are incubated with L-NIO (0.1μM) for 30 minutes before the incubation with 0, 5, 10 and 25 nM concentrations of leptin for 4 hours. 5 nM concentration of leptin shows a significantly higher level of NO release than other studied groups. Along with L-NIO, leptin shows

a significantly lower level of NO release as an indication of the demand of eNOS activation to release NO under leptin (n=6, mean $\pm$ SEM, *p<0.001 compared to control). (c). Comparison of NO-releasing ability of leptin with known NO agonist. EA.hy926 cells are incubated with 0, 5, and 10 nM concentrations of leptin, 10 ng of VEGF, and 1 μ M Bradykinin for 4 hours. Both the leptin concentrations show more or less equal potential on NO release compared to VEGF and Bradykinin (n=6, mean $\pm$ SEM, *p<0.001 compared to control). (d). Leptin induces a significant amount of NO release during the early incubation period – EA.hy926 cells are incubated with 0, 5, and 10 nM leptin for 0, 3, 6, and 240 minutes incubation. Leptin's 5 and 10 nM concentration shows a significant NO release at 3 and 6 minutes of incubation. (n=6, mean $\pm$ SEM, *p<0.001). (e). Direct measurement of NO release from ECs under leptin using a NO electrode. BAECs are incubated with 0, 5, and 10 nM concentrations of leptin and NO-releasing level is measured during the 6th and 12th minutes of incubation. Leptin of 5 nM concentration shows a higher peak in the NO release level early at 6 minutes of incubation. (n=4).

Figure 3

Human recombinant leptin translocates eNOS from the perinuclear membrane to the plasma membrane. (a). eNOS localization pattern for hours. ECV-304 eNOS GFP tagged cells are incubated with 0, 5, and 10 nM leptin separately for one and four hours. Images are recorded at 60X magnification using a DP71 color camera attached with an Olympus1X71 Epifluorescence microscope at 515 nm wavelength. Cells

incubated with 5 nM leptin show more eNOS localization at the plasma membrane for both the period. (n=6, the bar is 20 μ m, arrows indicate eNOS GFP presence, nucleus stained with DAPI). (b). Percentage of eNOS localization for hours. Cells are categorized into the plasma membrane bright and perinuclear membrane bright cells. Cells incubated with 5 nM concentration of leptin show a significantly higher percentage of (68%) of plasma membrane-localized cells during the 1st and 4th hour of the incubation period (n=6, mean $\pm$ SEM, *p=<0.001 compared to control). (c). Leptin translocates eNOS from the perinuclear membrane to the plasma membrane within minutes of incubation. ECV-304 eNOS GFP tagged cells are incubated with 0, 5, and 10 nM leptin concentrations for 3, 6, 9, and 12 minutes. Cells incubated with a 5 nM concentration of leptin show more eNOS localization at the plasma membrane at the 6th and 9th minutes of incubation. (n=6, the bar is 20 μ m, arrows indicate eNOS GFP presence, nucleus stained with DAPI). (d). Percentage of eNOS localization for minutes. Cells incubated with a 5 nM concentration of leptin show a significantly higher percentage of (69%) of plasma membrane-localized cells within 6 minutes of incubation as an indication of the potential of Nanomolar concentration leptin to induce eNOS subcellular localization within 6 minutes of incubation. (n=6, mean $\pm$ SEM, *p=<0.001 compared to control).

a

b

Figure 4

Presence of intracellular calcium level for the activation of eNOS. (a). Intracellular calcium level- Representative images of intracellular calcium level in ECs under leptin. EA.hy926 cells are incubated with 0, 5, and 10 nM of leptin for 6 minutes, followed by incubation with a 5 μ M Calcium green1/AM for another 30 minutes. Images are recorded using a DP71 camera attached to an Olympus 1X71 Epifluorescence microscope at 4X magnification. (n=4, bar is 10 μ m). (b). The average intensity of

calcium/cells. From the images, the average fluorescence intensity of the cells is calculated using Adobe Photoshop version 6. Cells treated with 5 nM leptin show significantly higher average intensity in intracellular calcium level ($n=4$, $^*p=0.05$, mean $\pm$ SEM).

Figure 5

Human recombinant leptin activates eNOS through phosphorylation at Serine 1177 residue. (a). Immunofluorescence analysis of co-localization of eNOS and phospho eNOS at Serine 1177. ECV-304 eNOS GFP tagged cells are incubated with 0 and 5 nM leptin for 6 and 9 minutes. Images are recorded at 60X magnification using a DP71 color camera attached to an Olympus 1X71 Epifluorescence microscope with required filters. Cells incubated with a 5 nM concentration of leptin show more co-localization at the plasma membrane for eNOS and phospho eNOS within 6 minutes of incubation. ($n=3$, the bar is 20 μ m, arrow indicates the presence of co-localization of eNOS and phospho eNOS, nucleus stained with DAPI). (b). Western blot analysis - ECV304 GFP tagged cells are incubated with 0 and 5 nM concentrations of leptin for 6 and 9 minutes. After the protein separation and wet blotting, the blots were developed, and the images are recorded using a BIO-RAD system. (c). Average protein level expression - Cells incubated with 5 nM leptin concentration within 6 and 9 minutes of incubation show the higher-level expression of eNOS and phospho eNOS (Serine 1177) significantly. The results indicate that leptin-induced eNOS activation depends on the phosphorylation of eNOS at Serine 1177 residue. ($n=3$, $p<0.001$, mean $\pm$ SEM).

Figure 6

Human recombinant leptin mediates the interaction of eNOS with Caveolin-1 at the plasma membrane. (a). Immunofluorescence analysis of co-localization of eNOS and Caveolin-1. ECV-304 eNOS GFP tagged cells are incubated with 0 and 5 nM concentrations of leptin for 6 minutes. After the overnight incubation, immunostaining was done with the primary antibody for Caveolin-1. Images are recorded at 60X magnification using a DP71 color camera attached to an Olympus 1X71 Epifluorescence microscope with required filters. Cells incubated with a 5 nM concentration of leptin show more co-localization at the plasma membrane for eNOS and Caveolin-1 within 6 minutes of incubation. (n=3, the bar is 20 μm, arrow indicates the presence of co-localization of eNOS and Caveolin-1, nucleus stained with DAPI). (b). Ultracentrifugation and western blot analysis. ECV-304 eNOS GFP tagged confluent cells are incubated with 0 and 5 nM leptin concentration for 6 minutes. After the ultracentrifugation, all the ten fractions are carefully collected and blotted for Caveolin-1 and eNOS expression. The images are recorded using a BIO-RAD system. The co-localization expression of Cav-1 and eNOS is higher at the plasma membrane fraction under 5 nM concentration of Leptin within 6 minutes of incubation. (1, 2, and 3 represent plasma fractions, 4, 5, and 6 represent Golgi fractions, and 7, 8, 9, and 10 represent nuclear membrane fractions).

Figure 7.c

Figure 7

The Nanomolar concentration of human recombinant leptin induce angiogenesis on early CAM. (a). In vivo angiogenic assay - Representative images of area vasculosa incubated with 0 and 5 nM leptin for 8 hours. Area vasculosa incubated with 5 nM leptin shows increased vasculature density than control (arrow). The effect is diminished under L-NAME. (n=3, bar is 10 μ m). (b). Quantification of angiogenesis - The length and the size of the blood vessel increased significantly under leptin incubation and decreased

in the presence of L-NAME (n=3, mean $\pm$ SEM, $\S p < 0.05$ compared to control). (c). Histological evaluation – The part of the area vasculosa incubated with 5 nM leptin concentration shows more angiogenic response than other studied groups. (n=3, bar is 50 μ m, LECS- large extracellular space; LBV- large blood vessels; V- vein; SBV- small blood vessel; BI- blood islands).

Supplementary Files

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