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Intralipid™ Increases Nitric Oxide Release from Human Endothelial Cells during Oxidative Stress

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Abstract

Background: Intralipid™, a clinically used lipid emulsion, has been shown to protect organs against ischemia/reperfusion (IR) injury. We hypothesized that Intralipid™ activates endothelial nitric oxide (NO) synthase (eNOS) and leads to increased NO release from endothelial cells (ECs) through a fatty acid translocase (CD36)-mediated endocytotic mechanism, which can act as a potentially protective paracrine signal during oxidative stress.

Methods: Human umbilical vein ECs were exposed to 1% Intralipid™ for 2 hours followed by oxidative stress with 0.2 mM hydrogen peroxide for 2 hours. Western blots were conducted with anti-CD36, dynamin-2, src-kinase-1, eNOS, and phospho-eNOS antibodies; equal protein loading was confirmed with β -actin. A CD36 immunoprecipitation assay was probed for caveolin-1 to determine if CD36 and caveolin-1 were complexed on the cell membrane. NO was measured by DAF fluorescence of ECs with and without Intralipid™.

Results: Intralipid™ caused a 227% increase in CD36 expression vs controls. Immunoprecipitation indicated that a CD36/caveolin-1 complex is present on the endothelial cell membrane with exposure to Intralipid™. Dynamin-2 increased by 52% and src-kinase-1 by 340% in cells treated with Intralipid™ vs control cells. eNOS phosphorylation was confirmed by a 63% increase in the phospho-eNOS / eNOS ratio in Intralipid™-treated cells, and NO fluorescence increased by 102%.

Conclusion: Intralipid™ enters ECs via endocytosis by a CD36/caveolin-1 cell membrane receptor complex, which in turn is pulled into the cell by dynamin-2 activity. Upregulation of

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Conflicts of interest: None

src-kinase-1 and eNOS phosphorylation suggest further downstream mediators. Subsequent NO release from ECs may serve as a paracrine signal to neighboring cells for protection against IR injury.

Keywords

CD36; endothelial cells; fat emulsion; ischemia/reperfusion injury; nitric oxide synthase; src-kinase-1

INTRODUCTION

Intralipid™ (ILP), a water-soluble fat emulsion consisting of fatty acids like linoleic (44-62%), oleic (19-30%), palmitic (7-14%), linolenic (4- 11%) and stearic (1.4-5.5%), has been clinically used to provide parenteral nutrition since 1962 ^{1, 2}. Additionally, ILP has been shown to counteract local anesthetic overdose and reduce the release of inflammatory mediators, postoperative complications in patients with major gastrointestinal surgeries, and IR injury ³.

Despite these advances in its clinical use, the mechanisms behind ILP's protective effects are far from clear, especially further downstream. During IR injury in the heart, it may be that endothelial cells (ECs) can serve as paracrine cells and enhance protective signaling in cardiomyocytes, as shown in a co-culture model of both cell types ⁴. However, in cultures of only isolated cardiomyocytes, direct exposure to certain free fatty acids comparable to ILP has been shown to be detrimental ⁵.

Fatty acid translocase, also known as CD36, is a transmembrane glycoprotein which is expressed in many different cell types, such as ECs ⁶, microglia cells, monocytes/macrophages ⁷, platelets ⁸, cardiac and skeletal muscle ⁹, adipocytes ¹⁰ and certain epithelial cells ¹¹. It is a multifunctional protein homologous to a scavenger receptor class B, type I. Its function is to facilitate fatty acid uptake ¹², especially playing a critical role in fatty acid uptake into ECs. CD36 may also play an important role in organ protection against oxidative stress, since recovery from IR injury is impaired in fatty acid translocase (CD36)-null mice ¹³, while addition of a CD36 ligand confers organ protection ¹⁴. Caveolae are cholesterol- and sphingolipid-enriched invaginations of the plasma membrane, with caveolins being structural proteins essential for the formation of caveolae. They are involved in endocytosis and downstream signal transduction ¹⁵. For example endocytosis of native LDL is facilitated by interaction of CD36 and caveolin-1 signaling in caveolae ¹⁶. Therefore, the objective of this study was to investigate if ILP enters ECs, and whether NO synthesis is enhanced/activated in response to ILP administration. We hypothesized that ILP is taken up into ECs via endocytosis following the formation of a CD36/caveolin-1 cell membrane complex, and that src-kinase-1 phosphorylates dynamin-2, a GTPase which acts to separate the vesicle from the plasma membrane. We further hypothesized that this leads to endothelial NO synthase (eNOS) activation and the production of the vasodilator and signaling molecule NO ¹⁷.

MATERIALS AND METHODS

Cell Culture and Experimental Preparation

Human umbilical vein ECs (Lonza, Williamsport, PA; passage #4-6, 80% confluent, n=4) were exposed to 1% ILP in cell culture media (20-fold dilution of a 20% emulsion; Fresenius Kabi, Uppsala, Sweden, culture media: EGM, Lonza) for 2 hours followed by oxidative stress provided by exposure to 0.2 mM hydrogen peroxide for 2 hours¹⁸. Total protein was isolated with RIPA lysis buffer and protein analysis was conducted by Bradford Assay.

Western Blot Analysis

Protein isolated from the ECs was probed for caveolin-1, eNOS, phospho-eNOS, src-kinase-1, phospho-src and dynamin-2 antibodies using Western blot analysis. Briefly, EC homogenates were separated on SDS-PAGE (4-20%) gels; equal protein loading onto each lane in the gel was confirmed with β -actin as a loading control. Nitrocellulose membranes containing the separated proteins were blocked in 10% nonfat dry milk solution in Tris-buffered saline (TBS). The blot was then incubated overnight using CD36 (sc-7309), caveolin-1 (sc-53564), eNOS (sc-376751), phospho-eNOS (sc-293032), src- (sc-32789) and phospho-src-kinase (sc-166860), dynamin-2 (sc-166669), and heat shock protein (Hsp) 90 (sc-13119) antibodies (1:1,000; Santa Cruz Biotechnologies Inc., Santa Cruz, CA) in TBS containing 5% nonfat dry milk. After washing four times, the blot was incubated with the horseradish peroxidase-conjugated donkey anti-mouse IgG for the Hsp90 antibody, anti-goat IgG for the dynamin-2 antibody, or anti-rabbit IgG for the remaining antibodies for 1hr (1:10,000 dilution in 2% nonfat dry milk in TBS; Santa Cruz Biotechnologies Inc.). Bands were visualized with ECL plus reagent using a Biorad ChemiDoc (Biorad, Hercules, Ca, GE Healthcare, Piscataway, NJ). Determination of relative band densities normalized to loading control was performed using image acquisition and analysis software (Image J, National Institutes of Health, Bethesda, MD). Westerns blots were run in triplicate.

Immunoprecipitation Assay

Immunoprecipitation assays of CD36 and eNOS were performed with the same antibodies used in the Western blot analyses, then immunoblotted for caveolin-1 and Hsp90, respectively. Briefly, the protein homogenate was precleared for 20 minutes with protein A agarose beads, followed by an antibody incubation overnight to bind the protein to be pulled down. Protein A beads were then added and bound to the antibody. After 2 hours of incubation, the beads were pelleted by centrifugation, the complex separated from the beads, and the beads removed. Western blots were performed as described above for each protein of interest (n = 4).

NO Detection by Fluorescence

4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF, 1:1,000, D23844, Invitrogen, Carlsbad, CA) fluorescence was measured in ECs to assess the effect of ILP treatment on NO production. Concomitant incubation of ILP with N-Nitro-L-Arginine Methyl Ester (L-NAME; 1mM concentration, N5751, Sigma Aldrich, St. Louis, MO), a

non-specific eNOS inhibitor, as a control was performed. Briefly, ECs were seeded on slides and exposed to 1% ILP as described above. The media was washed off, followed by a 30-minute incubation with DAF and a 15 minute wash in phosphate buffered saline (PBS). The slides were mounted in an aqueous mounting media (15148-062, Shur-Mount, VWR, Batavia, IL) and analyzed by confocal microscopy (Nikon Eclipse, Ez-C1, Melville, NY). The probes were visualized by confocal microscopy (Nikon Eclipse, Ez-C1) and analyzed with the Nikon Element Imaging software. (n = 4) as previously described ¹⁹.

Endosome Detection by Immunohistochemistry

ECs were treated with ILP as described above. To detect endosomes, the lysosome/endosome marker CD63/LAMP3 was used. The cells were then fixed with 1% paraformaldehyde and permeabilized with 0.5% Triton X-100. A 30-minute incubation at 37°C with the CD63/LAMP3 primary antibody (1:50, sc-5275, Santa Cruz Biotechnologies Inc.) was followed by three washes with PBS for 5 minutes each. The cells were then incubated with the secondary anti-rabbit antibody conjugated with Alexa 488 (1:50, A-11001, Invitrogen) for 30 minutes at 37°C, followed by three PBS washes for 5 minutes each. The styryl peptide FM 95-5 (1:50, T23360; Invitrogen) was then added for 10 minutes at room temperature. After three additional wash steps with PBS, the slide was mounted with aqueous mounting media. The probes were visualized by confocal microscopy (Nikon Eclipse, Ez-C1) and analyzed with the Nikon Element Imaging software (n = 4).

Statistics

All values are given as mean $\pm$ standard error or percent change over baseline. The Student's t-test was utilized for single comparisons and ANOVA with Bonferroni-Dunn post-hoc modification for multiple comparisons; $P < 0.05$ was considered statistically significant (two-tailed). Statistical symbols: * vs control, † vs ILP.

RESULTS

CD36 Expression Is Increased

To measure the fatty acid translocase CD36 expression, ECs were exposed to 1% ILP for 2 hours. The CD36 expression levels were 3.37-fold (*40,380 $\pm$ 1,721 arbitrary units, au) compared to control cells (11,991 $\pm$ 650 au) without ILP exposure (Figure 1A).

CD36 Forms a Complex with Caveolin-1

For endocytosis, CD36 forms a complex with caveolin-1. Immunoprecipitation of CD36 demonstrated complex formation with caveolin-1 and indicated that CD36/caveolin-1 protein complexes are more prevalent on the cell membrane of ECs after ILP exposure (Figure 1B). The association of caveolin-1 and CD36, as depicted by density of Western blot bands, increased from 16,385 $\pm$ 97 au in control cells to *36,534 $\pm$ 801 au in ILP-treated cells.

Expression of Dynamin-2 Is Increased

Dynamin 2 expression was assessed by Western blot analysis. Its expression was increased by 52% after ECs were exposed to 1% ILP for 2 hours ($32,523 \pm 1,731$ au) compared to ECs without ILP exposure ($21,442 \pm 1,225$ au) (Figure 2).

Src-kinase-1 Expression Is Increased

The role of Src kinase-1 is to facilitate the phosphorylation of dynamin-2. Src-kinase-1 expression was increased to 440% in ILP-treated *versus* untreated ECs, from $9,215 \pm 212$ au to $40,540 \pm 3,247$ au (Figure 3).

Increased Phosphorylation of eNOS and Increased Association with Hsp90

Activation of eNOS was assessed not only by phosphorylation but also by its association with Hsp90. In ECs treated with ILP, the ratio of phospho-eNOS to eNOS was increased by 63% as compared to ECs not exposed to ILP (Figure 4A). NO production from coupled eNOS activity was confirmed by a 96% increase in the eNOS/Hsp90 association when compared to ECs not exposed to ILP (Figure 4B).

ILP Increases NO in Endothelial Cells

Activation of eNOS by ILP was measured by NO fluorescence. DAF-fluorescence indicating NO production was increased by 102% in ECs exposed to ILP (448 ± 85 au) as compared to no treatment (222 ± 82 au); concomitant incubation of ILP with L-NAME prevented the increase in fluorescence intensity (124 ± 3 au) (Figure 5A).

ILP Increases the Formation of Lysosomes/Endosomes Intracellularly

CD63/LAMP3 and the styryl peptide FM5-95 are membrane markers for lysosomes/endosomes with distinctive binding sites. Two hours after ILP administration CD63/LAMP3 co-localized with the styryl peptide FM95-5 depicting lysosomes and endosomes in ECs (Figure 5B).

DISCUSSION

Developed by the Swedish physician Arvid Wretling in 1963, ILP is a water-soluble fat emulsion clinically used for parenteral nutrition for several decades¹. Made of soybean oil and egg yolk, it contains phospholipids, glycerol and a mixture of neutral triglycerides, with its major components being linoleic, oleic and palmitic acid²⁰. In addition, it is frequently used as a solvent for many lipophilic drugs that could otherwise not be injected intravenously²¹. And, thanks to the serendipitous landmark discoveries of lipid rescue therapy against local anesthetic-induced cardiotoxicity, first in rats²², then dogs²³, and later in a human patient by Rosenblatt et al.²⁴, ILP has recently gained a different indication, and is now found in practically every operating room and labor-delivery suite²⁵. Although acting as a “lipid sink” is a commonly accepted theory for its mechanism as an antidote against the systemic toxicity of local anesthetics²⁶ and other lipophilic drugs²⁷, recent reports on the attenuation of lipid rescue by the fatty-acid oxidation inhibitor CVT-4325²⁸

and the opioid receptor antagonist naloxone²⁹ suggest a more complex, possibly pathway-mediated mechanism²⁶.

The role of glucose *versus* fatty acid metabolism in attenuating IR injury has indeed been the subject of investigations and reviews^{30, 31}. It is entirely conceivable that fatty acids can serve as a more efficient fuel during IR³¹. On the other hand, metabolic modulators shifting metabolism preferentially to glucose utilization are a newer class of drugs to ameliorate IR injury³². Inhibition of fatty acid oxidation by etomoxir, for example, has been shown to be highly protective in hearts³³. Regardless of whether it is fuel switch- or receptor/pathway-mediated, the eventual targets of any protective strategy remain all the cells of the entire organ. Yet, it is not well understood if ILP, or any of its components, reach and act directly on the target cells, such as the cardiomyocytes of the myocardium, or if ILP's protective actions are *indirectly* mediated by the endothelium and endothelium-derived signals. However, interestingly, direct exposure of isolated cardiomyocytes to fatty acids has been shown to be detrimental⁵.

Despite the beneficial effects of ILP, an increase in fatty acids in the blood stream, and the impact on endothelial cells, can have a negative impact. Tampakakis et al.³⁴ reported in 2016 that a 5 hour infusion of ILP causes microvascular dysfunction and ER stress in healthy subjects via activating transcription factor 6 and phospho-inositol requiring kinase 1 by a TLR4 dependent pathway. Steinberg et al. report an impairment of insulin-mediated vasodilation and impaired NO production after elevating free fatty acids by an 8 hour exposure to ILP³⁵. In contrast, ILP is also described to modulate EC integrity by reducing LPS induced ICAM-1 expression and Nf κ b phosphorylation, hence reducing LPS induced inflammatory response³⁶.

The current investigation now focuses on the ability of ILP to induce cellular protection in states of high oxidative stress³⁷ by short term exposure of ECs to ILP. Despite substantial effort in this area, the exact mechanism by which ILP exhibits its protective effect remains elusive. Our findings strongly suggest an endocytotic pathway as depicted in Figure 9, visualizing how the mediators we investigated may interact. ILP increases the expression of CD36, the thrombospondin receptor also known as fatty acid translocase, in ECs³⁸. CD36 is crucial for fatty acid uptake into cells including ECs³⁹. Recovery from IR injury is impaired in fatty acid translocase (CD36)-null mice¹³, whereas addition of a CD36 ligand confers cardioprotection¹⁴. CD36 forms a complex with caveolin-1 and increases the src-kinase-1 expression. Src-kinase-1 activates dynamin-2, a GTPase essential for endocytosis, by phosphorylation, enabling it to be cleaved as vesicles from the membrane⁴⁰. Finally, this leads to coupling of eNOS through phosphorylation and association with Hsp90, and a subsequent increase in intracellular NO production from coupled eNOS. NO can then serve as a paracrine factor and initiate protective pathways in neighboring cells.

Some simplifications and limitations of our study need to be acknowledged. Cell cultures cannot completely replace a functioning organ, much less a complex *in vivo* model; they can, however, help answer questions that cannot be readily studied *in vivo*. By administering ILP directly to the endothelial cells, we attempted to mimic intravenous (IV) administration, assuming no substantial changes in composition and bioavailability that may occur *in vivo*

⁴¹. Instead of hypoxia, we simulated myocardial IR by exposure to hydrogen peroxide, an accepted model for oxidative stress in isolated cells ¹⁸. Furthermore, we describe a possible mechanism by which ILP leads to NO release through eNOS. Other authors, however, have reported the opposite, i.e., eNOS inhibition and endothelial dysfunction *in vivo* by fatty acids and ILP ⁴². The discrepancy in findings is more than likely based on the duration of ILP exposure. Lastly, oxidative stress in a cell culture model cannot substitute for the pathophysiological conditions and other potential confounders, such as diabetes, obesity, atherosclerosis, and/or congestive heart failure, under which organ IR typically occurs in patients.

Nevertheless, our study features several novel findings: i) stimulation of ECs with ILP results in an upregulation of CD36 and an increase in the association of CD36 with caveolin-1; ii) this is concomitant with increases in src-kinase-1 and dynamin-2 expression; iii) these changes in intracellular signaling lead to an increase in the number of endosomes (Figure 5B), iv) an activation of eNOS and v) an increase in NO production (Figure 4).

CONCLUSION

In summary, we report a novel receptor-mediated mechanism of how ILP, a clinically used lipid emulsion reported to be protective against IR injury, enters ECs via endocytosis by a CD36/caveolin-1 cell membrane receptor complex and increased dynamin-2 activity. Upregulated src-kinase-1 and phosphorylated eNOS, complexed with Hsp90, then lead to increased release of NO, which can potentially serve as a paracrine mediator for protection of neighboring cells against IR injury.

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Key Points

- **Question:** How does Intralipid™, a clinically used lipid emulsion, protect organ cells against oxidative stress?
- **Findings:** Intralipid™ enters endothelial cells via endocytosis and leads to increased nitric oxide production through endothelial nitric oxide synthase.
- **Meaning:** Intralipid™ may protect organ cells indirectly through increased endothelial production and release of nitric oxide as a paracrine mediator for neighboring cells.

Figure 1.

(A) *ILP increases CD36 expression.* Western blot analysis showed a significant increase in CD36 expression when ECs were exposed to 1% ILP for 2 hours compared to control cells; * $P < 0.05$ vs control. (B) *CD36 forms a complex with caveolin-1.* ECs exposed to 1% ILP showed a significantly increased association of CD36 and caveolin-1 as assessed by immunoblotting (IB) compared to ECs without ILP exposure; * $P < 0.05$ vs control.

Figure 2. Expression of dynamin-2 is increased.

Dynamin-2, a GTPase responsible for endocytosis, expression was significantly increased in ECs exposed to 1% ILP for 2 hours compared to controls cells; * $P < 0.05$ vs control.

Figure 3. ILP increases src-kinase-1 expression.

Src-kinase-1 expression was significantly increased in ECs exposed to 1% ILP for 2 hours compared to ECs not exposed to ILP; * $P < 0.05$ vs control.

Figure 4.

(A) **Increased phosphorylation of eNOS.** The ratio of phospho-eNOS to eNOS was significantly increased in ECs treated with 1% ILP for 2 hours as compared to ECs not exposed to ILP; * $P < 0.05$ vs control. (B) **ILP increases eNOS association with Hsp90.** NO production from coupled eNOS activity was confirmed by increased eNOS/Hsp90 association after treatment with 1% ILP when compared to ECs not exposed to ILP; * $P < 0.05$ vs control.

Figure 5.

(A) *ILP increases NO release.* NO production as assessed by DAF-fluorescence doubled in ECs exposed to 1% ILP for 2 hours. Concomitant incubation with L-NAME not only prevented this increase but brought it below control levels; * $P < 0.05$ vs control, † $P < 0.05$ vs ILP. (B) *ILP increases formation of endosomes:* CD63/LAMP3 fluorescence with ILP shows increased number of endosomes within the cell when compared to no ILP treatment; * $P < 0.05$ vs control.

Figure 6. Putative signaling mechanism by ILP.

Stimulation of ECs with ILP results in an upregulation of CD36 and an increase in the association of CD36 with caveolin-1, concomitant with increases in src-kinase-1 and dynamin-2 expression. These intracellular signals lead to a coupling of eNOS and increased NO production.