Intermittent hypoxia induces murine macrophage foam cell formation by IKK-β-dependent NF-kappa B pathway activation

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ABSTRACT

Obstructive sleep apnea (OSA) is a common sleep disorder characterized by intermittent hypoxia (IH). Clinical studies have previously shown that OSA is an independent risk factor for atherosclerosis. Atherogenicity in OSA patients has been assumed to be associated with the NF-κB pathways. Although foam cells are considered to be a hallmark of atherosclerosis, how IH as in OSA affects their development has not been fully understood. Therefore, we hypothesized that IH induces macrophage foam cell formation through NF-κB pathway activation. To test this hypothesis, peritoneal macrophages collected from myeloid-restricted IKK-β deleted mice were incubated with native LDL and exposed to either IH or normoxia. After exposure, NF-κB pathway activity and intracellular cholesterol were measured. In control macrophages, IH significantly increased NF-κB pathway activity by 93%
compared to normoxia ($P < 0.05$). However, such response to IH was diminished by
IKK-β deletion (increased by +31% compared to normoxia; $P = 0.64$), suggesting
that IKK-β is critical for IH-induced NF-κB pathway activation. Likewise, in control
macrophages, total cholesterol was increased in IH compared to normoxia (65.7 ±
3.8 μg/mg cellular protein and 53.2 ± 1.2, respectively; $P < 0.05$). However, this
IH-induced foam cell formation was disappeared when IKK-β was deleted (52.2 ±
1.2 μg/mg cellular protein for IH and 46.3 ± 1.7 for normoxia; $P = 0.55$). This
IH-mediated effect still existed in macrophages without LDL receptor. Taken
together, our findings show that IH activates the IKK-β-dependent NF-κB pathway
and that this, in turn, induces foam cell formation in murine macrophages.

NEW & NOTEWORTHY

IH-induced inflammation increases the risk of atherosclerosis in OSA
patients. However, the effect of IH on macrophage foam cell formation, a key player
in atherosclerosis, has not been elucidated. We demonstrate for the first time that
IH-induced foam cell formation is diminished by IKK-β deletion. Our findings
highlight the importance of the IKK-β-dependent NF-κB pathway and the potential
of this pathway as a therapeutic target.

INTRODUCTION

Obstructive sleep apnea (OSA) is a common sleep disorder predominant
in developed countries, including the United States. The prevalence of OSA in the
adult population in the U.S. was reported to be more than 10% (31, 43).
Interestingly, OSA patients not only experience sleep fragmentation and daytime
sleepiness, but also have increased risks of a variety of clinical conditions, such as
dyslipidemia, diabetes mellitus, and cardiovascular events (11, 35, 56). Furthermore,
OSA has been identified as an independent risk factor for atherosclerosis that
results in cardiovascular events (4, 12, 13).
One of the hallmark characteristics of OSA is intermittent hypoxia (IH).

From large-scale clinical studies, the risk of atherosclerosis seems to be correlated to the severity of OSA (20, 30). This link between IH and atherosclerosis was confirmed in animal experiments in which mice exposed to IH developed aortic atherosclerotic plaques (3, 14, 15, 23, 32, 49). The mechanism of such phenomenon is not yet fully understood. However, one clinical study has already demonstrated that the severity of the OSA is correlated with the serum level of TNF-α, which is dependent on NF-κB activity (47). Furthermore, from observations on human subjects, it has been documented that NF-κB activation contributes to atherosclerosis development (8). Taken together, it has been proposed that IH induces NF-κB activation, which in turn may contribute to atherogenicity in OSA patients (34, 38). This is further supported by rodent experiments in which IH-exposed mice on high-fat diet (HFD) developed less atherosclerosis when NF-κB p50 was deleted (15, 51).

To elucidate some of the complex mechanisms of atherosclerosis
development, it is essential to focus on cell-specific contribution. Macrophages seem to have an essential role in atherosclerosis development at all phases, from early fatty streaks to advanced lesions with necrotic cores (31). The finding that reduction of macrophages by CD11b-diphtheria toxin receptor or deletion of macrophage colony-stimulating factor ameliorates HFD-induced atherosclerosis in mice (50, 53) emphasizes the importance of macrophages in atherosclerosis.

Lipid-laden macrophages (foam cells) in the lesions are one of the earliest histological changes in atherosclerosis (52). Foam cells play a critical role in atherosclerosis development through activation of inflammatory pathways by secreting pro-inflammatory mediators including IL-6 and TNF-α and simultaneously recruiting immune cells and smooth muscle cells (17, 31). Therefore, it is essential to elucidate the effect of IH on foam cell formation to address the atherogenicity in OSA patients.

To date, a few researchers have documented IH-induced foam cell formation in macrophages and one implied the possible involvement of
inflammation (29, 32). Evidence indicates that IH activates the NF-κB pathways in HeLa cells (48). In addition, inhibition of these pathways by overexpression of IκBα results in a reduction of foam cell formation in murine peritoneal macrophages (16). Thus, we speculate that IH induces foam cell formation by NF-κB activation in macrophages.

IKK-β-dependent NF-κB activation has been especially implicated in human atherosclerosis (37). However, in spite of the importance of IH in atherogenicity, to our knowledge, no one has studied the role of IKK-β on IH-induced macrophage foam cell formation. Therefore, we hypothesized that IH exposure elicits IKK-β-dependent NF-κB activation, which in turn induces macrophage form cell formation. To test this hypothesis, we studied THP-1 cell line and IKK-β knockout mice and proved that deletion of IKK-β reduces IH-induced foam cell formation in murine peritoneal macrophages.

METHODS
Cell culture. THP-1 cells were originally obtained from American Tissue Type Cell Collection (ATCC, Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 40 μg/ml Gentamicin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA). THP-1 cells were seeded at a density of 2 × 10^6 cells/ml and incubated with 50 μM of phorbol 12-myristate 13-acetate (PMA) for 72 hours to induce differentiation into macrophages. After induction, the media was changed to low glucose (1 g/L) DMEM medium free of phenol red (11054-020, Gibco, Grand Island, NY) supplemented with Gentamicin and 10% FBS. We avoided phenol red because it is reported to have estrogenic activity (7) and estrogen inhibits foam cell formation in human monocyte-derived macrophages (36). The cells were incubated in a normoxia incubator for 24 hours before IH exposure.

Animals. Ldlr^-/- mice on C57BL/6J background were purchased (Stock number 002207; The Jackson Laboratory, Bar Harbor, ME). Ikk-beta^-/- mice were
kindly provided by Dr. Michael Karin at the University of California, San Diego, and myeloid-specific IKK-β knockout mice (*Ikk*-β\(^{ΔMye}\)) were generated as previously described (19). We crossed *Ikk*-β\(^{ΔMye}\) mice with *Ldlr\(^{-/-}\)* mice to generate *Ikk*-β\(^{ΔMye}\)-*Ldlr\(^{-/-}\)* double knockout mice. *Ikk*-β\(^{F/F}\) and *Ikk*-β\(^{F/F}\)-*Ldlr\(^{-/-}\)* mice were used as control animals. All animals used in this study were 2-3-month-old male mice fed with a regular chow diet.

This study was conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings and was approved by the University of California, San Diego, Institutional Animal Care and Use Committee (Protocol number: S-05534).

**Isolation of peritoneal macrophages.** Peritoneal macrophages were collected by peritoneal lavage with pyrogen-free PBS 3 days following peritoneal injection of 1 ml of 3% sterile thioglycolate (BD, Sparks, MD) to increase the yield of macrophages. The collected cells were incubated for 3 hours before the plates were washed twice with PBS to remove non-adherent cells. Macrophage monolayers
were then cultured overnight in phenol red-free low-glucose DMEM containing 1% FBS for lipid starvation. Before IH or normoxia exposure, media was changed to phenol red-free low glucose DMEM supplemented with different concentrations of native human LDL (BT-903, Alfa Aesar, Ward Hill, MA).

IH exposure. The incubator was specifically designed to periodically regulate oxygen concentration by controlling gas outlets of O₂, CO₂, and N₂ using a computerized system (LabVIEW, National Instruments, Austin, TX). The macrophages were exposed to IH in the incubator (25% O₂ for 8 min, 0% for 12 min, CO₂ was maintained at 5% throughout the exposure). One major pitfall of in vitro IH-exposure is the difficulty of generating the fluctuations of dissolved oxygen in media because of the relatively high resistance of the transport of oxygen from the air to cells. (6) Therefore, we validated the system by measuring the dissolved oxygen in media and confirmed its cyclic oscillation from 20.4%±0.12 to 2.76%±0.05 before starting (mean ± SD).

Lipid staining. Macrophages were plated onto Lab-Tek™ chambered glass
slides (Nalge Nunc, Naperville, IL), fixed with 4% paraformaldehyde for 15 minutes, and stained with Oil Red O and hematoxylin. Intracellular neutral lipid was measured by Nile red staining using the AdipoRed\textsuperscript{TM} Assay Reagent (Lonza, Walkersville, MD). The intensity of fluorescence was measured with excitation at 485 nm and emission at 530 nm.

**Cholesterol loading and quantification of intracellular cholesterol.** After differentiated THP-1 cells or peritoneal macrophages were loaded with cholesterol by incubation with 200 μg/ml of human native LDL for 24 hours to induce foam cell formation, the macrophages were washed twice with ice-cold PBS. The cellular lipids were extracted with hexane/isopropanol (3:2, v/v), as previously noted (22). Total cholesterol (TC) and free cholesterol (FC) were determined by the Amplex\textsuperscript{®} Red Cholesterol assay kit (Thermo Fisher Scientific, Waltham, MA). Cholesteryl ester (CE) was calculated by subtracting FC from TC. After lipid extraction, cellular protein was dissolved in 0.2 N sodium hydroxide and 1% SDS, and protein concentration was measured using the BCA assay (Sigma-Aldrich, St. Louis, MO).
Western blot analysis. Phosphorylated p-65, an indicator of activated NF-κB pathways, was performed using thioglycolate-elicited peritoneal macrophages. Macrophages were washed twice with ice-cold PBS, and nuclear protein was extracted by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents and Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA). The nuclear protein content was quantified and 25 μg was used for analysis. The membrane was incubated with the phospho-p65 antibody (#3033, Cell Signaling Technology, Danvers, MA). The results were normalized with HDAC1 (sc-7872, Santa Cruz Biotechnology, Dallas, TX) to correct for well-to-well variability.

Statistics. Data are reported as means ± SE. Differences between groups were analyzed using the Student’s unpaired t-test or Two-Way ANOVA with post-hoc Tukey’s multiple comparison test (GraphPad Prism, GraphPad Software, San Diego, CA) as appropriate. Differences in the means were considered statistically significant when \( P < 0.05 \).
RESULTS

Intermittent hypoxia induces foam cell formation in THP-1 cells. THP-1 human monocyte/macrophage-like cells were incubated in media with native LDL at various concentrations in either normoxia or IH for 24 hours (Fig. 1A). IH exposure induced a significant increase of lipid particles (Nile red staining) when 400 or 800 μg/ml of native LDL was added to the cultures (114 ± 4% in normoxia and 128 ± 3% in IH for 400 μg/ml, 127 ± 3% in normoxia and 140 ± 3% in IH for 800 μg/ml; values are percentages of the RFU of normoxia-exposed cells incubated without LDL; P < 0.05 for both comparisons, Fig. 1B). Remarkably, IH also significantly increased intracellular TC when cells were incubated with 200 μg/ml of native LDL (34.7 ± 1.2 μg/mg of cellular protein in normoxia and 40.1 ± 0.8 in IH; P < 0.05, Fig. 2A). Additionally, there was an IH-induced increase of CE (5.9 ± 0.4 μg/mg of cellular protein in normoxia and 8.9 ± 0.5 in IH for CE; P < 0.05, Fig. 2C). IH did not
Intermittent hypoxia activates the NF-κB pathway in peritoneal macrophages. To assess the effect of IH on the NF-κB pathways, peritoneal macrophages from \( \text{Ikk-}\beta^{F/F} \) and \( \text{Ikk-}\beta^{\Delta}\text{Mye} \) mice were incubated under either normoxia or IH. In the IH-exposed macrophages from \( \text{Ikk-}\beta^{F/F} \) mice, phosphorylated p-65 protein expression in the nucleus was increased by 93% compared to the normoxia-exposed \( \text{Ikk-}\beta^{F/F} \) macrophages (\( P = 0.05 \), Fig. 3). Interestingly, we observed no significant increase of NF-κB pathway activity in response to IH when IKK-\( \beta \) was deleted (\(+31\% \) activity increase in IH compared to normoxia; \( P = 0.64 \), Fig. 3). Therefore, we conclude that IH increases the NF-κB pathway activity and is dependent on pathways where IKK-\( \beta \) is a key component.

Intermittent hypoxia-induced foam cell formation depends on IKK-\( \beta \)-dependent NF-κB pathway. To investigate the role of IKK-\( \beta \)-dependent NF-κB activation in IH-induced foam cell formation, macrophages from \( \text{Ikk-}\beta^{F/F} \) and
Ikke-βΔMye mice were incubated with native LDL (Fig. 4) and assayed to quantify intracellular cholesterol after 24-hours in normoxia or IH (Fig. 5A-C). TC of IH-exposed macrophages from Ikke-βF/F was significantly increased compared to normoxia when they were incubated with 200 μg/ml of native LDL (53.2 ± 1.2 μg/mg cellular protein in normoxia and 65.7 ± 3.8 in IH; P < 0.05, Fig. 5A). However, IH did not increase TC when IKK-β was deleted (46.3 ± 1.7 μg/mg cellular protein in normoxia vs. 52.2 ± 1.2 in IH; P = 0.55, Fig. 5A). We also observed a significant, increase of FC in Ikke-βF/F macrophages exposed to IH as compared to normoxia (48.0 ± 1.2 μg/mg cellular protein in normoxia and 59.2 ± 4.3 in IH; P < 0.05, Fig. 5B), and there was no IH-induced FC increase in Ikke-βΔMye macrophages (40.6 ± 2.4 μg/mg cellular protein in normoxia and 46.4 ± 1.3 in IH; P = 0.52, Fig. 5B). CE was increased when the cells were incubated with LDL, but not increased by IH-exposure in both Ikke-βF/F and Ikke-βΔMye macrophages. In summary, IH increased intracellular TC in macrophages incubated with native LDL and showed a modest effect on FC, which was diminished by IKK-β deletion.
Cholesterol from non-modified native LDL is not taken up by LDL receptor-mediated internalization. To elucidate the role of uptake through LDL receptor in IH-induced foam cell formation, peritoneal macrophages from Ikk-β^{F/F}-Ldlr^{-/-} and Ikk-β^{ΔMye}-Ldlr^{-/-} mice were used. Intriguingly, we still observed the IH-induced TC increase that was diminished by IKK-β deletion (52.4 ± 1.7 μg/mg cellular protein in normoxia vs. 63.9 ± 4.8 in IH for Ikk-β^{F/F}-Ldlr^{-/-} and 45.8 ± 1.9 in normoxia vs. 47.9 ± 1.9 in IH for Ikk-β^{ΔMye}-Ldlr^{-/-}; P < 0.05 and P > 0.99, respectively, Fig. 6A), and a modest effect of IH on FC was observed again (45.5 ± 1.9 μg/mg cellular protein in normoxia vs. 53.2 ± 2.8 in IH for Ikk-β^{F/F}-Ldlr^{-/-} and 38.8 ± 2.1 in normoxia vs. 40.9 ± 1.5 in IH for Ikk-β^{ΔMye}-Ldlr^{-/-}; P = 0.09 and P > 0.99, respectively, Fig. 6B). CE was not increased by IH in both Ikk-β^{F/F}-Ldlr^{-/-} and Ikk-β^{ΔMye}-Ldlr^{-/-} macrophages. These findings indicate that the role of LDLR on IH-induced foam cell formation is not major and imply the presence of another mechanism, including increased cholesterol intake from native LDL by pinocytosis and inhibition of cholesterol efflux (18).
The results of this research show that IH activates the NF-κB pathways and induces foam cell formation. To the best of our knowledge, this is the first report to directly show that IKK-β-dependent NF-κB activation causes IH-induced macrophage foam cell formation. Our result is consistent with previously published evidence, researchers showed that IH exposure caused macrophage foam cell formation *in vitro* (28, 31). Furthermore, it has been shown that IH activates the NF-κB pathways in various types of cells (1, 21, 32, 41, 46, 48, 55). Other data demonstrated that activation of the NF-κB pathways contributed to foam cell formation where they employed PMA-treated THP-1 cells with inactive NF-κB/IκBα complex (16) and lipopolysaccharide-treated bone marrow-derived macrophages from NF-κB1 deficient mouse (24). Therefore, we speculated that IH causes NF-κB pathway activation, and in turn induces foam cell formation. In spite of the importance of OSA in atherogenicity, the IH-induced foam cell formation by NF-κB
pathway activation was not fully understood. In this study, we showed that the
IKK-β-dependent NF-κB activation is the pathway that causes IH-induced foam cell
formation by using IKK-β deleted mouse macrophages. Our results add essential
information to our understanding of the increased risk of cardiovascular disease in
OSA patients, which is now a big national health burden.

Evidence is growing to support the hypothesis that IH induces
atherosclerosis in mice on high fat diet (3, 13, 15, 23, 32, 49). First, we have indeed
shown that IH exposure induces foam cell formation in the THP-1 human
macrophage cell line as well as mouse peritoneal macrophages. Although
significance of macrophage foam cells, the characteristic of atherosclerotic lesions,
has been well established (31), only a few researchers have documented the
induction of foam cell formation by IH exposure (29, 32). One of the important
differences between our work and the work of others is that we supplemented the
culture medium with native LDL, which is a weaker inducer of foam cell formation
compared to chemically modified LDL, such as acetylated LDL, that was used by
other investigators (29, 32). Since the use of chemically modified LDL for
cholesterol-loading experiments has been previously criticized (26, 27), we used
200 to 800 μg/mg of native LDL. This range of LDL concentrations is equivalent to
10 to 40 mg/dl in plasma, a range that is considered normal in humans. It is very
interesting that we were still able to observe the IH-induced effect on intracellular
cholesterol metabolism in spite of using relatively low concentrations of
non-modified LDL.

One of the questions that can be raised is how cholesterol enters
macrophages to produce foam cells. Of interest is that previous research has
shown that activated macrophages by PMA or M-CSF increase their native LDL
uptake through fluid-phase pinocytosis (2, 27, 28, 59). Thus, we believe that IH
likely works to mediate LDL uptake by this receptor-independent mechanism since
IH continues to induce cholesterol accumulation in macrophages even when LDLR,
which is the major receptor for LDL internalization, is absent.

The NF-κB pathways have two major activation signaling processes,
canonical and non-canonical. IKK-β is the prevalent catalytic subunit of the IKK complex that is necessary for the canonical pathway of NF-κB activation (25).

IKK-β-dependent NF-κB activation has been implicated in human atherosclerosis (37) and the importance of IKK-β in the pathogenesis of atherosclerosis has been well documented (5, 39). An important finding in our work is that we show that IH activates IKK-β-dependent NF-κB pathways in murine peritoneal macrophages because our IKK-β deleted macrophages in IH did not activate the NF-κB pathways and did not accumulate more cholesterol compared to normoxia. This indicates that IKK-β-dependent pathway is essential for IH-induced NF-κB activation and foam cell formation.

One very important question to answer is related to the mechanisms of how IKK-β-dependent NF-κB activation regulates intracellular cholesterol metabolism. Cholesterol homeostasis in macrophages is regulated by many biochemical steps (31). Briefly, native LDL is taken up by LDLR or via pinocytosis (17, 26). Whereas, modified LDL, such as acetylated or oxidized LDL, is chiefly
taken up by scavenger receptors (38). Then, internalized cholesterol is stored as CE (9, 18). As an initial step in the efflux, CE is hydrolyzed to form FC (18, 40).

Lastly, ABCA-1 and ABCG-1 mediate cholesterol efflux, which is facilitated by SR-BI (45). Since FC can induce inflammation and cause cytotoxicity (33, 54, 60), macrophages are normally protected from accumulation of excess FC because it can be injurious to them. Remarkably, IH significantly increased intracellular FC in the peritoneal macrophages from mice, an effect that was not observed when IKK-β was deleted. We observed a significant increase of CE but not FC in THP-1 cells. The reason for this difference could be explained by the difference in intracellular cholesterol metabolism between human and mouse (10), or possibly between cell lines and primary cells (41).

Lipid assays of lesions from various stages of human and animal atherosclerosis reveal a progressive intracellular FC increase in macrophages as lesions become more advanced (44). Since it has been reported that constant hypoxia also increases intracellular FC in murine macrophage cell lines and bone
marrow derived macrophages (42), further investigations are required to address
whether IH and constant hypoxia share similar mechanisms to increase FC, and
furthermore, the role of IKK-β-dependent NF-κB pathways on this.

There are a few limitations to our study. Firstly, we used
thioglycolate-elicited macrophages. Although many researchers in this field have
used these types of macrophages, it is known that this agent itself can induce
inflammation (58). However, there was no difference in NF-κB pathway activity
between \(\text{Ikk-β}^{F/F}\) and \(\text{Ikk-β}^{Δ\text{Mye}}\) macrophages in normoxia. Bone marrow-derived
macrophages could be an alternative method, but M-CSF that is used to
differentiate cells into macrophages is also known to augment macrophage
pinocytosis and induce foam cell formation (59). Secondly, IKK-β deletion in this
model was reported not to cause complete deletion (approximately 75% reduction
of the expression; 19). Nonetheless, we were still able to observe a significant
reduction of NF-κB pathway activation in the IH-exposed macrophages when IKK-β
was deleted. Thirdly, we did not address detailed mechanisms of this IH-induced
foam cell formation by IKK-β-dependent NF-κB activation in this study. Fourthly, while the uptake of native LDL is through LDLR or micropinocytosis, but modified LDL is taken up through scavenger receptors, such as SRA, CD36, and LOX-1 (38). Our aim in this paper was not how to assess whether oxidized LDL was taken up through these scavenger receptors. Lastly, we did not assess in this study the mechanisms of uptake or the correlation between IH-induced foam cell formation and atherogenicity in OSA patients, and in vivo animal experiments will be necessary to address this.

In summary, we have demonstrated that IH induces IKK-β-dependent NF-κB activation in macrophages and that inflammation is important for foam cell formation. Our findings have implications for the relation between OSA and macrophage foam cell formation through NF-κB pathway activation. Our results could potentially provide therapeutic targets to reduce the risk of cardiovascular disease in OSA patients.
ACKNOWLEDGEMENTS

We thank Dr. Michael Karin at University of California, San Diego for providing Ikk-β<sup>F/F</sup> mice.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


**FIGURE CAPTIONS**

Fig. 1. Effects of IH on intracellular lipids in THP-1 cells. A: Microscopy images of Oil red O stained THP-1 cells incubated with different concentrations of native LDL. The cells were exposed to either normoxia or IH for 24 hours. B: Quantification of intracellular neutral lipids by Nile Red staining. (Values are expressed as means ± SE; n = 6; *P < 0.05 vs. normoxia)

Fig. 2. Effects of IH on intracellular cholesterol in THP-1 cells. A: Total cholesterol, B: Free cholesterol, C: Cholesteryl ester. (Values are expressed as means ± SE; n = 10; *P < 0.05 compared to IH-exposed cells incubated with 200 μg/dl native LDL)
Fig. 3. IKK-β-dependent NF-κB activation by IH in peritoneal macrophages. The activity of the NF-κB pathway was measured as phosphorylated p65 in nuclear protein. Representative data of Western blot (upper panel) and summary data (lower panel). (Values are expressed as means ± SE; n = 5; *P < 0.05 compared to IH-exposed Ikk-βF/F macrophages)

Fig. 4. Microscopy images of Oil red O stained peritoneal macrophages from Ikk-βF/F and Ikk-βΔMye mice incubated with different concentrations of native LDL. The cells were exposed to either normoxia or IH for 24 hours.

Fig. 5. Effects of IH and deletion of IKK-β on intracellular cholesterol contents in peritoneal macrophages from Ikk-βΔMye and Ikk-βF/F mice where A: Total cholesterol, B: Free cholesterol, and C: Cholesteryl ester. (Values are expressed as means ± SE; n = 7; *P < 0.05 vs. IH-exposed Ikk-βF/F macrophages incubated with 200 μg/dl native LDL)
Fig. 6. Effects of IH and deletion of IKK-β on intracellular cholesterol contents in peritoneal macrophages from lkk-β^ΔMye^-Ldlr^−/− and lkk-β^F/F^-Ldlr^−/− mice where A: Total cholesterol, B: Free cholesterol, and C: Cholesteryl ester. (Values are expressed as means ± SE; n = 7; *P < 0.05 vs. IH-exposed lkk-β^F/F^-Ldlr^−/− macrophages incubated with 200 μg/dl native LDL)
A

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B

![Graph](image9.png)

**% RFU of normoxia without LDL**

- **Normoxia**
- **Intermittent hypoxia**

**Concentration of native LDL**

- no LDL
- 200 µg/ml
- 400 µg/ml
- 800 µg/ml
**Native LDL**

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**Ikk-β\textsuperscript{F/F}**

**Ikk-β\textsuperscript{ΔMye}**
Macrophages from Ikk-β\(^{F/F}\) and Ikk-β\(^{ΔMye}\)

**A** Total cholesterol

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**B** Free cholesterol

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**C** Cholesteryl ester

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Macrophages from Ikk-β^{F/F}-Ldlr^{-/-} and Ikk-β^{ΔMyc}-Ldlr^{-/-}

A  Total cholesterol

B  Free cholesterol

C  Cholesteryl ester

* indicates a significant difference.

- **A**: Total cholesterol levels in macrophages from Ikk-β^{F/F}-Ldlr^{-/-} and Ikk-β^{ΔMyc}-Ldlr^{-/-} under different conditions.
- **B**: Free cholesterol levels.
- **C**: Cholesteryl ester levels.

Comparison of cholesterol content in different conditions:

- **Baseline**: No LDL, 200 μg/ml LDL.
- **Norm**: Normal conditions.
- **IH**: Inflammatory conditions.

**Notes**:
- μg cholesterol / mg of cellular protein
- * indicates significant difference.