Nucleotides released from palmitate challenged muscle cells through pannexin-3 attract monocytes

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Abstract

Obesity-associated low-grade inflammation in metabolically relevant tissues contributes to insulin resistance. We recently reported monocyte/macrophage infiltration in mouse and human skeletal muscles. However, the molecular triggers of this infiltration are unknown, and the role of muscle cells in this context is poorly understood.

Animal studies are not amenable to specifically investigate this vectorial cellular communication. Using cell cultures, we investigated the crosstalk between myotubes and monocytes exposed to physiological levels of saturated and unsaturated fatty acids. Media from L6 myotubes treated with palmitate – but not palmitoleate – induced THP1 monocyte migration across transwells. Palmitate activated the TLR4-NFκB pathway in myotubes and elevated cytokine expression, but the monocyte chemoattracting agent was not a polypeptide. Instead, nucleotide degradation eliminated the chemoattracting properties of the myotube conditioned-media. Moreover, palmitate-induced expression and activity of pannexin-3 channels in myotubes was mediated by TLR4-NFκB, and TLR4-NFκB inhibition or pannexin-3 knockdown prevented monocyte chemoattraction. In mice, the expression of pannexin channels increased in adipose tissue and skeletal muscle in response to high fat feeding.

These findings identify pannexins as new targets of saturated fatty acid-induced inflammation in myotubes, and point to nucleotides as possible mediators of immune cell chemoattraction towards muscle in the context of obesity.
Introduction

Nutrient excess is a major factor contributing to the alarming incidence of obesity world-wide (1). Obesity leads to whole-body insulin resistance, a leading cause of type 2 diabetes and cardiovascular complications (1). A paradigm shift in our understanding of the inflammation accompanying obesity was the discovery that high fat diet (HFD) increases the amount of immune cells in adipose tissue, and a growing body of evidence now suggests that obesity and type 2 diabetes are inflammatory diseases (2). Surprisingly, equivalent studies in skeletal muscle had been relatively scant in spite of this tissue being responsible for the majority of postprandial glucose utilization (3).

We and others recently demonstrated an increase in macrophage number and inflammatory phenotype within skeletal muscle from HFD-fed mice and obese subjects (4, 5), but strikingly, the factors responsible for immune cell infiltration in obese muscle (or other metabolically-relevant tissues) are largely unknown. Skeletal muscle secretes several cytokines, recently renamed “myokines” (6), but the specific soluble mediators, channels and receptors involved in the crosstalk between skeletal muscle and immune cells are virtually undefined. Interestingly, in addition to myokines, selective stimuli induce the release of small molecules from muscle, such as prostanoids, lactate and nucleotides (7–12). The mechanism of release of these small molecules is unclear, but likely occurs through channels expressed at the plasma membrane. Pannexins are recently discovered channel-forming proteins that allow the release of cytoplasmic molecules to the extracellular space (13, 14). Whereas pannexin-1 and its role in physiological release of small molecules has been widely studied (15–17), the functions of
pannexin-2 and pannexin-3 remain elusive. Moreover, the contribution of pannexin channels and small molecule release during metabolic inflammation remains unexplored.

Although the initial trigger of inflammation in vivo is debated, excess lipids contribute to the induction of insulin resistance and of pro-inflammatory genes in metabolic tissues (18). Compellingly, palmitic acid (hexadecanoic acid, 16:0), a major dietary saturated fatty acid in blood, promotes a pro-inflammatory phenotype in several cell types in vitro (19–22). On the other hand, unsaturated fatty acids such as the monounsaturated fatty acid palmitoleic acid ((Z)-9-hexadecenoic acid, 16:1Δ9) are either innocuous or able to suppress inflammation (22, 23).

Given the diversity of cells coexisting within metabolic tissues (parenchymal, endothelial and myeloid) it is difficult to dissect the particular role of myocytes in the immune cell infiltration of muscle tissue during HFD. Only cell culture paradigms allow for the establishment of vectorial communication between distinct cell types and their response to defined hyperlipidic environments. Here we show that when myotubes are challenged with palmitate – but not with palmitoleate – they release non-peptidic factors that attract monocytes. We provide evidence that nucleotides released through pannexin-3 are major factors in this palmitate-induced crosstalk between muscle and immune cells.
Research Design and Methods

Reagents. MYD88 inhibitory peptides were from Invivogen (San Diego, CA). siRNAs oligonucleotides for connexin-43, connexin-45 and TLR4 were from GenePharma (Shanghai, China) and for pannexin-3 from Qiagen (Chatsworth, CA). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

Palmitate preparation. Palmitate or palmitoleate (P9767 and P9417, Sigma Aldrich) stock solutions (200mM) were prepared in 50% ethanol by heating at 50°C. Fatty-acid free, low-endotoxin BSA (A8806, Sigma Aldrich) was dissolved in serum-free αMEM to 10.5%. Fatty acid stocks were diluted 25X in the BSA solution and conjugated under agitation at 40°C for 2h. This solution (lipid:BSA ratio 5:1) was further diluted in culture media. Palmitate and palmitoleate solutions thus coupled to BSA are denoted as PA and PO, respectively.

Cell culture, viability and transfection. L6 muscle cells were grown and differentiated as described previously (24). THP1 monocytes were grown in RPMI-1640 containing 5% FBS. Cellular viability was assessed from LDH activity and MTT reduction (Cytotoxicity Detection Kit and Cell Proliferation Kit I, Roche Applied Science, Indianapolis, IN). Oligonucleotide siRNAs were transfected with JetPRIME (Polyplus-transfection, Illkirch, France). Myotubes were treated with 200nM siRNA for 24h, then stabilized in fresh media for 24h before experiments.

Generation of muscle conditioned medium. L6 myotubes were treated in αMEM (2% FBS) for doses and times indicated. Supernatants were centrifuged at 10,000 RPM for 5min to pellet debris, aliquoted and frozen immediately at -80°C. Supernatants (conditioned media) from PA,
PO and BSA-treated myotubes are herein denoted as CM-PA, CM-PO and CM-BSA, respectively.

**Fatty acid uptake.** Non-esterified fatty acids were quantified in the media of fatty acid-incubated myotubes using the NEFA-HR kit (Wako Chemical, Richmond, VA). Uptake was indirectly estimated from the fatty acid content in the myotube supernatant at the beginning and end of the incubation.

**Monocyte chemoattraction assay.** In Boyden chambers (Transwell, 6.5mm diameter, 5µm pore diameter; Corning, Lowell, MA), 600µL of attractant were added to the lower chamber. THP1 monocytes (100µL of 5x10⁶ cells/ml) in αMEM supplemented with 2% FBS were placed in the upper chamber. After 3h at 37°C, cells were dislodged from the filter by gentle shaking, the upper chamber was discarded and monocytes that transmigrated to the bottom well were counted using a Z2 coulter counter (Beckman Coulter Canada, Mississauga, ON).

**Nucleotide measurements.** ATP was specifically measured using the luciferase-based ENLITEN® ATP Assay (Promega, Madison, WI). Other nucleotides (mono-, di- and tri-phosphate) and nucleosides were measured by hydrophilic interaction liquid chromatography (HILIC) coupled to mass spectrometry as previously reported (25). The method was modified by limiting the monitored metabolites to nucleosides and nucleotides. Supernatants were analyzed by a Shimadzu Nexera UPLC (Shimadzu Corporation, Japan) coupled to AB/SCIEX Triple Quad 5500 mass spectrometer (AB/SCIEX, Framingham, MA) using electrospray ionization technique (ESI) operating in multiple reactions monitoring mode (MRM). Parent to product transitions employed for each detected metabolite are presented in **Supplemental Table 1.** Calibration curves (12.5-500 ng/mL), were generated for each detected metabolite. UTP, ATP, TDP, TTP,
CDP and GTP were undetectable (limit of detection: 1ng/mL). Standard of UDP was not available at the time of analysis and consequently results were reported in relative units by comparing the UDP peak areas.

**RNA isolation & qPCR.** All reagents were from Life Technologies (Carlsbad, CA). RNA was isolated using Trizol and cDNA synthetized using the SuperScript® VILO™ cDNA kit. Ten ng per reaction were used for RT-qPCR using pre-designed Taqman probes for target genes and hprt1 or eef2 (housekeeping references).

**Immunoblotting.** Cells were scraped in lysis buffer and protein content measured using BCA assay. Samples were boiled in Laemmli buffer, separated by SDS-PAGE and transferred onto nitrocellulose. Membranes were blotted using primary and peroxidase-coupled secondary antibodies, then developed using chemiluminescence (ECL, Bio-Rad, Hercules, CA), and analyzed using ImageJ software (NIH, Bethesda, MD).

**YO-PRO uptake.** YO-PRO (1µM) and Texas-red dextran (10kDa, 0.1mg/mL) from Life Technologies were added to cells for 15min. Cells were then washed with PBS and fixed (3% PFA, 10min). Images were acquired with a Leica DMIRE2 fluorescent microscope 10X air objective. Total green and red fluorescence were measured on 15 random fields per condition using ImageJ software.

**Multiplex Cytokine Analysis.** Cytokines were determined with a rat Milliplex MAP Magnetic bead panel (Millipore, Hellerup, Denmark) on a Bio-Plex-200 System (Bio-Rad Laboratories, Copenhagen, Denmark).

**Animal studies.** The study was approved by The Hospital for Sick Children Animal Care Committee. Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, MA), singly caged and
maintained at 21-22°C with light from 6:00-18:00, were fed a standard chow diet (5P07 Prolab RMH 1000, LabDiet, St. Louis, MO) or a high fat (60% by kcal) diet (D12492, Research Diets, New Brunswick, NJ) for 18 weeks. Following a 4h fast, mice were euthanized via cervical dislocation, tissues were collected, flash frozen in liquid nitrogen and preserved at -80°C.

**Statistical analysis.** Analyses were performed using Prism software (GraphPad Software, San Diego, CA). Results from dose-responses were compared by two-way analysis of variance (dose, treatment) followed by Tukey post hoc-tests. One-way ANOVA was used to test differences between groups with equal variances. Statistical significance was set at p<0.05.
Results

Palmitate-treated myotubes attract monocytes

Palmitate is the most abundant saturated fatty acid in western diets and it is widely used to challenge cells in culture. L6 myotubes were treated with BSA-conjugated palmitate (PA) or with the equivalent 16-carbon chain length monounsaturated palmitoleate (PO). Thereafter, supernatants (conditioned-media, CM) were collected and their chemoattracting activity towards THP-1 monocytes was determined (Fig.1A-B). Conditioned-media from PA-treated myotubes (CM-PA) had a marked chemoattracting effect that was not reproduced by conditioned-media from either PO- or BSA-treated myotubes (CM-PO and CM-BSA, respectively). Maximal chemoattraction was observed with treatment with 0.5mM PA for 18h. As the conditioned-media still contain fatty acids, monocyte migration was also measured towards regular medium containing 0.5mM fatty acids (Fig.1C). Unlike the chemoattracting chemokine CCL2/MCP1 (100ng/mL), neither PA nor PO on its own significantly affected monocyte migration. Uptake of PA and PO by myotubes was similar (Fig.1D), and an equivalent reduction in fatty acid and glucose content in the conditioned-media was observed in all conditions (Supplementary Tables 2 and 3). These results indicate that monocyte chemoattraction by CM-PA was not mediated by the fatty acid itself or by differences in myotube uptake of either fatty acids or glucose.

Palmitate-dependent myotube-induced chemoattraction of monocytes requires the TLR-MYD88-NFκB pathway
Toll-like receptors (TLR) couple with adaptor MYD88 to activate the NFκB transcription factor, a critical step in palmitate-induced inflammatory response (18, 21). In myotubes, PA evoked a dose-dependent decrease in IκB, the canonical NFκB repressor (Fig.2A). Preventing IκB degradation in myotubes with parthenolide or a cell-permeant MYD88 inhibitory peptide (Fig.2B-C) blunted the increase in monocyte migration induced by CM-PA. The involvement of this pathway was further confirmed by gene silencing. With an achieved reduction in expression of TLR4 and MYD88 of 65% and 80%, respectively, the CM-PA-evoked monocyte chemoattraction was abolished (Fig.2D). Together, these experiments demonstrate that PA engages the TLR-MYD88-NFκB pathway in myotubes to attract monocytes.

The chemoattractant released by myotubes is not a polypeptidic cytokine

The expression of chemokines and cytokines, classical monocytes attractants, was analyzed using qPCR arrays (Fig.3A). In myotubes exposed to PA, but not to PO, there was a significant rise in gene expression of several chemokines (Cxcl2, Ccl2, Cxcl1) and cytokines (Il1a, Tnfa, Il6), but surprisingly, that did not translate into their corresponding elevated secretion into the medium as measured by Luminex multiplex immunoassay (Fig.3B). This was confirmed using a membrane cytokine array and ELISAs for TNFα and CCL2 (Supplementary Fig.S1). To explore whether the chemoattracting factor would be affected by conditions altering protein stability, CM-PA and CM-BSA were either heated or treated with proteinase K (Fig.3C). These manipulations did not prevent the monocyte migration induced by CM-PA, suggesting that the chemoattractant is unlikely to be a polypeptide. Furthermore, upon filtering the conditioned-
media through a Vivaspin column, only the fraction containing molecules <3,000Da displayed monocyte chemoattracting activity (Fig.3C).

As TLR4 signalling in myotubes was involved in the monocyte chemoattracting ability of CM-PA (Fig.2), we explored whether activation of TLR4 signaling with LPS could elicit similar effects. Conditioned-media from LPS-treated myotubes (CM-LPS) also induced monocyte migration but interestingly, and unlike CM-PA, the monocyte chemoattracting activity of CM-LPS was heat- and proteinase K-sensitive and retained in the fraction containing molecules >3,000Da (Fig.3D). Blocking antibodies neutralizing the chemokine CCL2/MCP1 did not affect CM-PA-induced monocyte migration (Fig.3E), but completely prevented monocyte migration towards CM-LPS (Fig.3F). Together, these results suggest that in response to palmitate, the myotube-derived factor(s) responsible for monocytes migration is a low molecular weight compound. In contrast, CCL2/MCP1 is the monocyte chemoattractant released by myotubes in response to LPS.

Nucleotides are released by myotubes and are potent monocyte chemoattractants

Small molecules such as eicosanoids and nucleotides are bona fide regulators of the immune response and can potentially affect monocyte migration (26, 27). We consequently tested the chemoattractant effect of a variety of such small molecules. Eicosanoids (prostaglandins E2 and F2α and arachidonic acid) and histamine were monocyte repellents, urate and lactate had no effect, and only CCL2/MCP1, formyl peptides (WKYMVdM) and ATP were able to attract monocytes (Fig.4).
Nucleotides were the only small molecules that induced higher monocyte transmigration than CM-PA, with the potency order UTP>ATP>ADP>UDP>TTP (Fig.5A-B). The attracting ability of CM-PA was eliminated when nucleotides were hydrolyzed with the nucleotidase apyrase (Fig.5C). Notably, using an ATP-specific luciferase-based assay, ATP levels were detected and found to be higher in CM-PA compared to CM-PO or CM-BSA, and, consistent with the blunted monocyte attraction, apyrase treatment eliminated any measurable ATP in the conditioned-media (Fig.5D).

Most cells express surface extracellular ecto-nucleotidases that cleave nucleotides from the interstitial space (28). Accordingly, we explored whether endogenous ecto-nucleotidases would tonically reduce the amount of chemoattractant released by the myotubes. Inhibition of myotube ecto-nucleotidases with ARL67156 augmented the levels of ATP (Fig.5E) and concomitantly potentiated the monocyte chemoattracting activity of CM-PA (Fig.5F), without affecting either parameter in CM-BSA or CM-PO. Compellingly, the ATP concentration in CM-PA correlated strongly with monocyte chemoattraction (r=0.747, p<0.0001) across treatments and conditions (Fig.5G), buttressing the proposition that nucleotides are responsible for the enhanced monocyte transmigration towards CM-PA.

Blocking the P2 family of receptors on monocytes using the broad-spectrum non-selective antagonists suramin and PPADS also prevented CM-PA-induced monocyte chemoattraction (Fig.5H). However, since P2 receptors can be activated by several nucleotides (29) and apyrase can cleave all nucleotide tri- and di-phosphates (Fig.5I), the results suggested that, in addition to ATP, other nucleotides may also be involved. Indeed, using hydrophilic interaction liquid chromatography coupled to mass spectrometry, we found elevated concentrations of ADP, UDP
and several monophosphate nucleotides in CM-PA compared to CM-BSA and CM-PO (Fig.5J-K).

Since cytotoxic effects of fatty acids have been described in several cell types, and cells can release ATP after membrane damage, we assayed myotube viability. Neither MTT reduction, LDH release or caspase-3 cleavage was significantly affected in myotubes treated with PA compared to BSA or PO (Supplementary Table 4 and Fig.S2). Moreover, after mechanically damaging myotubes by scraping and vortexing to induce necrosis, LDH release increased 15-fold, indicating that more than 95% of the cells were undergoing necrosis. However, contrary to palmitate treatment, the supernatant from these scraped cells did not attract monocytes. In addition, no correlation was found between LDH release and monocyte chemoattraction (Supplementary Fig.S2). Thus, it is unlikely that the monocyte chemoattracting activity of CM-PA was due to sporadic myotube cell death.

Nucleotides are released from myotubes through pannexin-3 channels

The route of ATP release from myotubes was next examined. Pannexins are channels that allow the release of cytoplasmic molecules into the extracellular space (14). In addition, gap junction molecules (connexins) can form hemichannels, also allowing the release of small molecules (13). In L6 myotubes, connexin-43 (Cx43) and connexin-45 (Cx45) were the most abundant connexins (Fig.6A) while connexin-40 and pannexin-2 were undetectable. Notably, only the expression of pannexin-3 significantly rose with PA treatment (Fig.6B), and this effect was blocked by inhibiting NFκB with parthenolide (Fig.6C).
In order to mediate ATP release, channels must be open at the plasma membrane. The small dye YO-PRO®-1 has a molecular weight (630 g/mol) close to that of ATP (507 g/mol), readily diffuses through open connexin/pannexin channels and fluoresces upon binding to nucleic acids; hence, it has been used to ascertain the presence of open channels at the cell surface (15). YO-PRO was administered to myotubes treated with PA, PO or BSA, along with a large Texas-Red-dextran (10 kDa) that cannot go through connexin/pannexin channels but enters cells with damaged membranes (Supplementary Fig. S3). In myotubes treated with PA, the fluorescence ratio of YO-PRO/Texas-Red-dextran was significantly higher than in PO- or BSA-treated myotubes (Fig. 6D), indicating that PA increases the amount of open channels at the myotube plasma membrane.

Next, we reduced the expression of channels using cognate siRNA sequences. Only the knockdown of pannexin-3 eliminated the CM-PA-induced monocyte transmigration (Fig. 6E). Consistently, the ATP content significantly diminished in CM-PA derived from pannexin-3-depleted myotubes (Fig. 6F). These results demonstrate that pannexin-3 is required for nucleotide release by PA-treated myotubes into the media and for the consequent monocyte chemoattraction.

**Pannexin channel expression rises in high fat feed mice and palmitate-treated primary human myotubes**

Finally, in a pilot experiment we measured the expression of pannexins in epidydimal adipose tissue (eWAT) and quadriceps muscle from a small cohort of mice fed a high fat diet for 18 weeks. Pannexin-1 was highly expressed in both tissues and increased in response to high fat feeding in eWAT but not quadriceps (Fig. 7A-B). Expression of pannexins 2 and 3 was lower, but high fat feeding induced a significantly higher expression in eWAT. In particular, pannexin-3
expression in this tissue was undetectable in chow-fed mice and was noticeably induced by high-fat feeding (Fig.7B). In quadriceps muscle, the trend was very limited and more mice will be needed for proper statistical analysis of the results. Of note, in human primary myotubes, pannexin-2 expression rose significantly in response to palmitate (Fig.7C). These results demonstrate that although pannexin isoforms may differ, palmitate-induced expression of pannexin channels is common across species and relevant during high fat feeding in mice.

Discussion

Recent studies document a rise in macrophages within skeletal muscle of HFD-fed mice and obese individuals (4, 5, 30–33), but a key unresolved issue is whether and how skeletal muscle is capable of attracting monocytes. Here, we present evidence that conditioned-media from myotubes exposed to the saturated fatty acid PA, but not the unsaturated PO, attract monocytes in culture. Myotubes challenged in this manner activate endogenous inflammatory programs leading to the expression of pannexin-3 channels, and we identify nucleotides as new potential factors in muscle to monocyte crosstalk during metabolic inflammation.

In vivo, down-regulation of CCL2/MCP1 (34) or its receptor CCR2 (35) only partially prevented the inflammatory macrophages gain in adipose tissue and skeletal muscle of HFD-fed mice (5), suggesting that additional factors contribute to macrophage infiltration of tissues. In addition, two important studies recently ascribed the gain in adipose tissue macrophages to in situ proliferation, which was dependent on CCL2/MCP1 (36, 37). Hence, the chemoattracting function of CCL2/MCP1 in obesity is debatable. Even when exerting a chemoattracting effect,
CCL2/MCP1 might have been produced in response to endotoxin-like stimuli. Finally and importantly, it is not possible to ascertain from the above *in vivo* studies whether CCL2/MCP1 arose from myocytes/adipocytes, or whether instead it was contributed by endothelial cells or myeloid cells inside the tissues. It is precisely to approach these questions that we here explored the ability of a reconstituted cellular system to deconstruct the potential crosstalk between muscle and immune cells in the presence of fatty acids. We show that L6 myotubes selectively challenged with PA can attract monocytes, and that this does not rely on any chemokine. Instead, the chemoattracting factors are nucleotides released through pannexin-3 channels. By contrast, LPS-treated myotubes provoked a CCL2/MCP1-dependent monocyte chemoattraction. This suggests that both nucleotides and CCL2/MCP1 might contribute to the immune cell infiltration of skeletal muscle *in vivo* that occurs during high fat diets. Finally, primary human myotubes also showed increased pannexin expression in response to palmitate, as did tissues from high fat-fed mice.

The inflammatory response of myotubes to saturated fat leads to expression of pannexin-3

Dietary fats, in particular saturated ones, confer a state of low-grade inflammation to skeletal muscle. Either TLR activation or intracellular lipid intermediates can trigger an inflammatory response through activation of stress kinases (JNK, ERK), generation of reactive oxygen species and stimulation of NFκB signalling, classically enhancing expression of pro-inflammatory cytokines (38, 39). However, there is no previous evidence of inflammatory cues regulating the expression of channels for small molecules. We report here that activation of the TLR4-MYD88-NFκB pathway in myotubes challenged with palmitate significantly elevates the
expression of pannexin-3. This response was not observed when exposing myotubes to the unsaturated fatty acid palmitoleate, and therefore represents a nutrient-specific response. In particular, we observed a significant rise in several nucleotides in the conditioned-media from palmitate-challenged myotubes, and silencing the expression of pannexin-3 abolished this gain.

**Nucleotide release from myotubes: a ‘find me’ signal for monocytes**

The importance of extracellular nucleotides in cell-to-cell communication is evident in the immune system, where released ATP acts as a 'find-me' signal for immune cells to promote phagocytic clearance of damaged or apoptotic cells (27). Specific release of ATP also occurs in skeletal muscle during physiological exercise as well as during pathological situations such as sepsis or myopathies (For review, see (40)). Attempts to measure interstitial nucleotides using microdialysis have been scant, but have estimated ATP concentrations around 1µM in skeletal muscle from anesthetized cats (41) and around 0.1µM in humans (42). Studies in other tissues reported interstitial ATP in the nM range (43, 44) but none of these experiments could take into account the ATP concentration in the unstirred layer covering the cell surface (45). Overall, the basal interstitial concentration of ATP can be estimated in the 1-100nM range, but in pathological situations, extracellular ATP concentration can raise markedly to reach up to 10µM, as recently observed in tumor microenvironment (46). In contrast, it is unknown whether extracellular/interstitial nucleotide levels change during obesity-associated inflammation.

Although significantly higher than the level of ATP detected in CM-PO and CM-BSA, the concentration of ATP in CM-PA reached 10nM, a concentration which was insufficient to induce monocyte migration. However, the monocyte chemoattracting activity of CM-PA was
abolished by apyrase, and we corroborated that apyrase cleaves all nucleotides tri-phosphates. While the vast majority of studies on physiologically released nucleotides have focussed on ATP, UTP is also released during cardiac ischemia (47) and is a potent inducer of cell migration (48). Since CTP, GTP and TTP could not be detected in CM-PA (assayed with a detection limit of 2nM), and more than 1µM of each is required for effective chemoattraction (Fig.5), these nucleotides are unlikely to participate in the CM-PA-induced chemoattraction. Hence, we surmise that a combination of ATP, ADP, UDP and/or UTP constitute the chemoattracting ‘find me’ signal for monocytes towards palmitate-challenged muscle cells. Consistent with this assertion, monocytes express several P2 receptors that selectively recognize nucleotides. Even though all of them respond to ATP, P2Y2 and P2Y4 have high affinity for UTP; P2Y6 and P2Y8 for UDP; and P2Y1, P1Y12 and P2Y13 for ADP (29). As two broad-spectrum non-selective antagonists of the P2 receptors, suramin and PPADS, reduced the CM-PA-induced monocyte attraction, it is conceivable that nucleotides released by PA-challenged myotubes enact chemoattracting activity by activating one or more P2X/P2Y receptors on monocytes.

**Implications for inflammation associated with metabolic disease**

The results described in this study bring a new understanding to the lipotoxic inflammatory response of myotubes. Along with our previously reported inflammatory polarization of macrophages conferred by palmitate-treated muscle cells (22), these results illustrate the bidirectional crosstalk that occurs between muscle and immune cells in the context of hyperlipidic environments. Such bidirectional communication could only be ascertained through the described use of defined cell culture paradigms.
In addition to the palmitate-induced increase in pannexin-3 in L6 myotubes and pannexin-2 in human myotubes, our pilot in vivo results suggest that pannexins 2 and 3 are upregulated in eWAT from obese mice (and there might be a trend towards increase in quadriceps muscle). The response in tissues is more complex than in cells, as muscle and adipose tissue are composed of various cell types and express different pannexin isoforms. Moreover, increasing the expression of pannexin is not the only way to induce nucleotide release, as opening of the channels can also be regulated. Animal models will be needed in the future to provide deeper insight into the role of nucleotides and pannexin channels in diet-induced obesity.

Irrespective of whether changes in pannexins expression occur in muscle with obesity, our results show a previously unrealized principle, that treatment of myotubes with PA results in the expression of pannexin-3 through the TLR-NFkB pathway, and consequent release of nucleotides to the medium to chemoattract monocytes (Fig.8). Whether increases in pannexin expression and/or pannexin opening are required needs to be explored, but our siRNA results show that the existence of pannexin channels is required for nucleotide release and monocyte chemoattraction.

In conclusion, these findings constitute proof of concept of muscle to monocytes communication in hyperlipidic environments, and raise the possibility that in vivo, muscle fibers might also release nucleotides through pannexins to promote macrophage infiltration of skeletal muscle. Our findings would also predict that targeting pathways responsible for chemokine production may be insufficient to reduce macrophage infiltration of muscle, as other factors such as nucleotides may play a significant role in immune cell chemoattraction. Finally, our studies point to pannexins as interesting targets to taper the recruitment of tissue inflammatory macrophages during metabolic disease.
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Author contribution

NJP participated in the design of the study, coordinated and carried out the majority of the experiments, performed the statistical analysis and wrote the manuscript. YEL helped with experiments and analysis concerning muscle signalling and monocyte migration. LNF performed the Luminex multiplex analysis. JTB, AN, and MSK performed the liquid chromatography–mass spectrometry analysis. PJB participated in the design of the study, helped with the experiments and writing of the manuscript. AK conceived the study and participated in its design, coordination, and writing of the manuscript. All authors read and approved the final manuscript. The authors declare no conflict of interest. AK is the guarantor of this work and, as such, had full
access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References


47. Erlinge D et al. (2005) Uridine triphosphate (UTP) is released during cardiac ischemia. *Int J Cardiol* 100:427–433.

Figure Legends

Figure 1: Conditioned-media from palmitate-treated muscle cells attracts monocytes. A) Migration of THP1 monocytes toward conditioned-media from L6 myotubes treated for 18h with PA (CM-PA), PO (CM-PO) or BSA (CM-BSA). B) Transmigration of THP1 monocytes toward conditioned-media from L6 myotubes treated with 0.5mM fatty acids for 0, 3, 6 and 18h. C) CCL2/MCP1 (100ng/mL) was used as positive control and the effect of PA and PO on migration was controlled by placing 0.5mM fatty acids in the bottom chamber and testing THP1 monocyte transmigration. D) Fatty acid uptake by L6 myotubes treated for 18h was measured as described in Methods. All results are the mean ± SEM, n≥4, *p<0.05, **p<0.01 vs BSA control.

Figure 2: Inhibition of TLR-NFκB signaling in muscle prevents ATP release and monocyte migration. A) Activation of the NFκB pathway was measured by the degradation of its repressor IκB in L6 myotubes treated for 18h with palmitate (PA), palmitoleate (PO) or BSA vehicle. Immunoblotting was performed using specific antibodies to IκB. Blots were quantified and densitometry results expressed relative to actinin-1 as a loading control. Results are the average of 7 independent experiments and a representative gel is illustrated. B-C) L6 myotubes were pretreated with the NFκB inhibitor parthenolide (PTN, 25µM for 1h) or with an MYD88 inhibitory peptide (Pep-MYD, 50µM for 3h) and then treated with PA, PO or BSA for 18h in presence of the same inhibitor. D) Myotubes were transfected with siRNA to silence TLR4 or MYD88 as described in Methods. PA (0.5mM) or BSA were then added to the media for 18h. Conditioned-media were collected and THP1 monocyte migration was measured as described in Methods.
Insert: Efficiency of gene silencing measured by qPCR. Results are mean ± SEM, n≥4, **p<0.01 vs BSA control, ns: not significant.

**Figure 3: The attractant released by muscle cells is not a chemokine.** L6 myotubes were treated with 0.5mM PA or PO for 18h. A) Cytokine and chemokine expression was analyzed using qPCR arrays. B) Cytokine and chemokine content in myotube conditioned-media was analyzed by Luminex multiplex immunoassay. C) Conditioned-media from myotubes was heat-inactivated (95°C, 20 min) or treated with proteinase K (PROK, 100µg/mL for 1.5h at 37°C) to denature proteins. Untreated conditioned-media was also filtered through a 3-kDa cut-off membrane and the included and excluded fractions were tested separately for monocyte chemoattraction activity. D) Myotubes were treated for 18h with 100ng/mL LPS. Conditioned-medium (CM-LPS) was then collected and tested in a migration assay as described above. E-F) CM-PA and CM-LPS were treated with blocking antibodies against CCL2/MCP1 or IgG control. Results are the mean ± SEM, n≥4, *p<0.05, **p<0.01 vs BSA control. N.D: not detectable.

**Figure 4: Compounds affecting monocyte migration across transwells.** Chemokines (CCL2/MCP1 and CXCL1), formylated peptides (WKYMvdm and fMLP), ceramides (C2, C8 and C8-1-phospho sphate), eicosanoids (arachidonic acid ARA, PGE2 and PGF2α), lactic and uric acids, histamine and ATP were tested in dose-response for their ability to attract THP1 monocytes. Results are the average of at least 4 independent experiments and data were fit to a non-linear sigmoidal dose-response curve.

**Figure 5: Palmitate induces nucleotide release by muscle cells to attract monocytes.** A-B) Migration of THP-1 monocytes towards graded doses of purine and pyrimidine nucleotides. C-D) Conditioned-media from PA- and PO-treated muscle cells were treated with apyrase (0.2 UI/mL
final) for 1 h. **E-F**) Myotubes were treated with 0.5mM BSA/PA in the presence of the ecto-nucleotidase inhibitor ARL67156 (100µM) for 18h. The ATP content and chemoattraction activity of the corresponding conditioned-media were then measured. **G**) Correlation between ATP concentration in conditioned-media and monocyte migration. **H**) CM from myotubes was supplemented with the P2 receptor antagonists suramin (100µM) and PPADS (200µM) before testing monocyte migration. **I**) Apyrase (0.2 UI/mL final for 1h) was added to solutions containing nucleotide triphosphates at the concentration that caused half-maximal stimulation of monocytes chemoattraction for each nucleotide before testing monocyte migration. **J-K**) Nucleotides were measured using liquid chromatography coupled to mass spectrometry. Monocyte migration and ATP concentration were measured as described in Methods. Results are the mean ± SEM, n≥4, *p<0.05, **p<0.01 vs BSA control, ns: not significant.

**Figure 6: Nucleotides are released through pannexins-3 channels.** L6 myotubes were challenged with 0.5mM PA, PO or BSA for 18h. **A-B**) The expression of several pannexins and connexins was measured by qPCR. **C**) Myotubes were treated with NFκB inhibitor parthenolide (25µM) during the fatty acid challenges, and then pannexin-3 expression was determined. **D**) Uptake of YO-PRO was used as index of channel opening as described in Methods. **E-F**) Silencing Cx43 and Cx45 and Pan3 in myotubes was performed using siRNA oligonucleotides (200nM), conditioned-media were collected and their THP1 monocyte chemoattracting activity or ATP concentration was determined. Insert: Efficiency of transfection measured by qPCR. Results are the mean ± SEM, n≥4, *p<0.05, **p<0.01, ND: not detectable, ns: not significant.

**Figure 7: Pannexin channel expression rises with high fat diet in mice and upon palmitate exposure in human myotubes.** **A-B**) Male C57BL/6J mice were fed a high fat diet for 18 weeks. Expression of pannexins in quadriceps muscle and epidydimal adipose tissue (eWAT) was
measured by RT-qPCR. Results are the mean, n≥4. Dotted line represents the threshold of detection. C) Primary human myotubes were treated with 0.5 mM palmitate, palmitoleate or the BSA control for 24h. Expression of pannexins was measured using RT-qPCR. Results are the mean ± SEM, n≥4, *p<0.05, **p<0.01, ns: not significant vs control, ND: not detectable. Since chow samples were undetectable for Panx3, Wilcoxon test was used, setting the hypothetical value at the detection threshold.

**Figure 8:** Schematic representation of how nucleotides are released through pannexin channels from fatty-acid challenged muscle cells and attract monocytes. Activation of the TLR4-NFκB pathways leads to an increase in pannexin expression and opening at the plasma membrane. The subsequent nucleotide release attracts monocytes. Figure created using Servier Medical Art (http://www.servier.com).
Fig. 1

A) THP-1 migration (% of RM control) versus Concentration (mM)

B) THP-1 migration (% of RM control) versus Time (hours)

C) THP-1 migration (% of RM control) for various conditions

D) Fatty acid uptake (µmol/h) versus Initial conc. (mM)
**Fig. 2**

A. **IkBα/actinin-1 (relative to control)**

- BSA
- PA
- PO

B. **THP-1 migration (% of RM control)**

- DMSO
- PTN

C. **THP-1 migration (% of RM control)**

- Pep-CTL
- Pep-MYD

D. **THP-1 migration (% of RM control)**

- si-MyD88
- si-TLR4
- si-NR

**Concentration (mM)**

- 0.0
- 0.2
- 0.4
- 0.6
- 0.8

**IkBα**

**Actin-1**

**Concentration (mM)**

- 0.0
- 0.2
- 0.5
- 0.8

**ATP (nM)**

- 0
- 2
- 4
- 6
- 8

**% silencing**

- 0%
- 30%
- 50%
- 70%
- 100%

**p=0.15**
A  Quadriceps

B  eWAT

C  Primary human myotubes

Diabetes
Fig. 8

Diabetes

Myotube

Panx

p65

Nucleotides

Migration

Monocyte

Palmitate

MYD88

IκB

p65

Transcription

Nucleotides

P2X/R / P2Y/R

Migration
Supplementary Fig. S1: Palmitate does not affect the release of cytokines and chemokines from L6 myotubes. L6 myotubes were treated with 0.5mM PA or PO for 18h. Cytokine and chemokine content in myotube conditioned-media was analyzed using a cytokine array (Rat Cytokine Array C2, RayBiotech) and concentration of TNFα and MCP1/CCL2 in supernatant was confirmed using ELISA immunoassays (Rat quantikine, R&D systems), following manufacturer’s instructions.
**Fig. S2**: Necrosis and apoptosis do not correlate with monocyte migration. A) L6 myotubes were treated with PA, PO or BSA control for 18h. Caspase-3 was then measured in cell lysate using specific antibody recognizing both the pro- and cleaved forms of caspase-3. A representative blot is show. B) L6 myotubes were mechanically damaged by scraping and vortexing to induce necrosis. Conditioned media was then collected, centrifuged to pellet debris and tested for monocyte attraction as described in methods. Mean ± SEM, n=4. C) LDH release was plotted against CM-induced monocyte migration. Results are the mean from 18 independent experiments.
Supplementary Fig. S3

Fig. S3: YoPro uptake into myotubes in response to fatty acids. L6 myotubes differentiated in 6-well plates were treated with 0.5 mM BSA, PA or PO for 18 h as described in Methods. YoPro dye (1 µM) and Texas-red dextran (10 kDa, 0.1 mg/mL) were added for 15 min, then cells were washed 4x in ice-cold PBS, fixed with 3% PFA for 10 min and washed again with PBS. Images were taken with a Leica DMIRE2 fluorescence microscope using the 10X air objective. Total green and red fluorescence were measured using ImageJ software in 15 random fields per condition.
Supplementary Table 1. MRM transitions and ESI polarity mode used for detection of AMP, ADP, UDP, GMP, CMP, UMP, IMP and adenosine.

<table>
<thead>
<tr>
<th>Component</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Polarity mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>348</td>
<td>136</td>
<td>positive</td>
</tr>
<tr>
<td>ADP</td>
<td>426</td>
<td>159</td>
<td>negative</td>
</tr>
<tr>
<td>UDP</td>
<td>403</td>
<td>159</td>
<td>negative</td>
</tr>
<tr>
<td>CMP</td>
<td>324</td>
<td>112</td>
<td>positive</td>
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<tr>
<td>GMP</td>
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<tr>
<td>IMP</td>
<td>349</td>
<td>137</td>
<td>positive</td>
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<tr>
<td>Adenosine</td>
<td>268</td>
<td>136</td>
<td>positive</td>
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Supplementary Table 2. Measured NEFA concentration

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Supernatant</th>
<th>Change</th>
<th>Filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM</td>
<td>25 ± 25</td>
<td>29 ± 34</td>
<td>+16 %</td>
<td>&lt; 10</td>
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<tr>
<td>0.2 mM</td>
<td>BSA</td>
<td>15 ± 14</td>
<td>24 ± 26</td>
<td>+57 %</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>197 ± 58</td>
<td>90 ± 21</td>
<td>-54 %</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>252 ± 80</td>
<td>112 ± 24</td>
<td>-56 %</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>BSA</td>
<td>18 ± 17</td>
<td>28 ± 26</td>
<td>+56 %</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>478 ± 73</td>
<td>242 ± 36</td>
<td>-49 %</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>442 ± 119</td>
<td>299 ± 44</td>
<td>-32 %</td>
</tr>
<tr>
<td>0.8 mM</td>
<td>BSA</td>
<td>34 ± 35</td>
<td>49 ± 34</td>
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<tr>
<td></td>
<td>PA</td>
<td>723 ± 136</td>
<td>438 ± 58</td>
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<tr>
<td></td>
<td>PO</td>
<td>737 ± 222</td>
<td>467 ± 72</td>
<td>-37 %</td>
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</table>

NEFA concentrations in the initial media, myotube conditioned-media and filtrates (<3000 Da) were measured using the enzymatic method based on Acyl-CoA oxidase described in Methods. Results are means ± SD expressed in micromoles/litre from at least 4 independent experiments (n≥4).
Supplementary Table 3. Measured glucose concentration

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Supernatant</th>
<th>Change</th>
<th>Filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM</td>
<td>6.7 ± 0.4</td>
<td>3.9 ± 0.6</td>
<td>-46 %</td>
<td>3.5 ± 0.2</td>
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<tr>
<td>BSA</td>
<td>6.6 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>-39 %</td>
<td>4.0 ± 0.2</td>
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<tr>
<td>PA</td>
<td>7.0 ± 0.1</td>
<td>3.5 ± 0.4</td>
<td>-48 %</td>
<td>3.8 ± 0.2</td>
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<tr>
<td>PO</td>
<td>6.4 ± 0.6</td>
<td>3.5 ± 0.7</td>
<td>-43 %</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>

Glucose concentration in the initial media, conditioned-media and filtrates from L6 myotubes treated for 18h with 0.5 mM palmitate, palmitoleate or BSA control. Concentrations were measured using a glucometer. Results are means ± SD, expressed in mM from at least 4 independent experiments (n≥4).

Supplementary Table 4. Viability of L6 myotubes

<table>
<thead>
<tr>
<th></th>
<th>0.2 mM</th>
<th>0.5 mM</th>
<th>0.8 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.97 ± 0.08</td>
<td>0.75 ± 0.05</td>
<td>0.90 ± 0.07</td>
</tr>
<tr>
<td>PA</td>
<td>0.95 ± 0.03</td>
<td>0.99 ± 0.08</td>
<td>1.22 ± 0.04*</td>
</tr>
<tr>
<td>PO</td>
<td>0.85 ± 0.10</td>
<td>0.79 ± 0.04</td>
<td>1.05 ± 0.10</td>
</tr>
<tr>
<td>MTT reduction</td>
<td>0.2 mM</td>
<td>0.5 mM</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>BSA</td>
<td>0.79 ± 0.02</td>
<td>0.75 ± 0.05</td>
<td>0.73 ± 0.05</td>
</tr>
<tr>
<td>PA</td>
<td>0.73 ± 0.09</td>
<td>0.54 ± 0.04</td>
<td>0.45 ± 0.07*</td>
</tr>
<tr>
<td>PO</td>
<td>0.79 ± 0.07</td>
<td>0.70 ± 0.05</td>
<td>0.64 ± 0.04</td>
</tr>
</tbody>
</table>

The potential cytotoxicity caused by fatty acid treatment was estimated through LDH release and MTT reduction after exposing L6 myotubes to BSA, PA and PO for 18h. Results are normalized to the regular media control and expressed as mean ± SD, n ≥4. * p < 0.05 vs BSA control.