Relevance of the MEK/ERK Signaling Pathway in the Metabolism of Activated Macrophages: A Metabolomic Approach

Paqui G. Través, Pedro de Atauri, Silvia Marín, María Pimentel-Santillana, Juan-Carlos Rodríguez-Prados, Igor Marín de Mas, Vitaly A. Selivanov, Paloma Martín-Sanz, Lisardo Boscá and Marta Cascente

J Immunol published online 21 December 2011
http://www.jimmunol.org/content/early/2011/12/21/jimmunol.1101781
Relevance of the MEK/ERK Signaling Pathway in the Metabolism of Activated Macrophages: A Metabolomic Approach

Paqui G. Través,* Pedro de Atauri,†‡§,† Silvia Marín,†‡§,† María Pimentel-Santillana,* Juan-Carlos Rodríguez-Prados,† Igor Marín de Mas,†‡ Vitaly A. Selivanov,†‡ Paloma Martín-Sanz,*,†§ Lisardo Boscá,*§ and Marta Cascante*†‡

The activation of immune cells in response to a pathogen involves a succession of signaling events leading to gene and protein expression, which requires metabolic changes to match the energy demands. The metabolic profile associated with the MAPK cascade (ERK1/2, p38, and JNK) in macrophages was studied, and the effect of its inhibition on the specific metabolic pattern of LPS stimulation was characterized. A [1,2-13C2]glucose tracer-based metabolomic approach was used to examine the metabolic flow distribution in these cells after MEK/ERK inhibition. Bioinformatic tools were used to analyze changes in mass isotopemer distribution and changes in glucose and glutamine consumption and lactate production in basal and LPS-stimulated conditions in the presence and absence of the selective inhibitor of the MEK/ERK cascade, PD325901. Results showed that PD325901-mediated ERK1/2 inhibition significantly decreased glucose consumption and lactate production but did not affect glutamine consumption. These changes were accompanied by a decrease in the glycolytic flux, consistent with the observed decrease in fructose-2,6-bisphosphate concentration. The oxidative and nonoxidative pentose phosphate pathways and the ratio between them also decreased. However, tricarboxylic acid cycle flux did not change significantly. LPS activation led to the opposite responses, although all of these were suppressed by PD325901. However, LPS also induced a small decrease in pentose phosphate pathway fluxes and an increase in glutamine consumption that were not affected by PD325901. We concluded that inhibition of the MEK/ERK cascade interferes with central metabolism, and this cross-talk between signal transduction and metabolism also occurs in the presence of LPS. The Journal of Immunology, 2012, 188: 000–000.

MACrophages have important roles in innate and acquired immunity, as well as in tissue homeostasis (1, 2). Their activation is a complex process involving signaling events triggered by multiple inflammatory mediators, including exogenous factors, such as LPS, and endogenous mediators, such as cytokines and chemokines. Cytokines are major regulators of macrophage activation that limit the amount of inflammation and, thus, prevent toxicity and tissue damage (3, 4). Failure to induce an inflammatory response promotes unrestricted microbial proliferation and the development of serious infections, whereas excessive production of proinflammatory mediators may also be life threatening, as observed in patients with severe sepsis or septic shock. Therefore, immune responses must be tightly regulated (3, 5, 6).

MEK/ERK signaling is involved in the activation of oxidative and nitrosative bursts, endosomal trafficking, and proinflammatory macrophage polarization (1, 3, 7–9). Therefore, MEK/ERK signaling is likely to enhance macrophage activity against intracellular pathogens (10–12). The MEK/ERK pathway in macrophages is one of the most widely studied intracellular signaling cascades involved in LPS-induced proinflammatory responses (10). In addition to this, the effect of inhibition of p38 and JNK with the selective inhibitors BIRB796 and BI78D3, respectively, has been evaluated (12, 13).

NF-kB and MAPK signaling pathways (ERK, JNK, and p38) play a key role in the activation and regulation of innate and adaptive immune responses. For example, macrophages activate MEK/ERK cascade in response to bacterial infection. MEK/ERK signaling is involved in the activation of oxidative and nitrosative bursts, endosomal trafficking, and proinflammatory macrophage polarization (1, 3, 7–9). Therefore, MEK/ERK signaling is likely to enhance macrophage activity against intracellular pathogens (10–12). The MEK/ERK pathway in macrophages is one of the most widely studied intracellular signaling cascades involved in LPS-induced proinflammatory responses (10). In addition to this, the effect of inhibition of p38 and JNK with the selective inhibitors BIRB796 and BI78D3, respectively, has been evaluated (12, 13).

Address correspondence and reprint requests to Dr. Lisardo Boscá or Dr. Marta Cascante, Instituto de Investigaciones Biomédicas “Alberto Sols,” Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain (L.B.) or Department of Biochemistry and Molecular Biology, Faculty of Biology, Universitat de Barcelona, Edifici Nou, Planta-2, Avinguda Diagona l645, 08028 Barcelona, Spain (M.C.). E-mail addresses: lbosca@iib.uam.es (L.B.) and martacascante@ub.edu (M.C.)

Abbreviations used in this article: COX-2, cyclooxygenase 2; DAF-2DA, 4,5-diamino-fluorescein diacetate; DCFH-DA, dichlorofluorescein diacetate; FBPase-2, fructose-2,6-bisphosphatase; Fru-1,6-P2, fructose-1,6-bisphosphate; Fru-2,6-P2, fructose-2,6-bisphosphate; G0PDH, glucose-6-phosphate dehydrogenase; L-PFK-2, liver-type-PFK-2; Mal, malate; NOS-2, NO synthase 2; Oaa, oxaloacetate; Pdh, pyruvate dehydrogenase; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; 6PGDH, 6-phospho-D-glucuronate dehydrogenase; PI, propidium iodide; PPP, pentose phosphate pathway; Pyr, pyruvate; ROS, reactive oxygen species; TCA, tricarboxylic acid; uPFK-2, uPFK-2 isoenzyme of PFK-2.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00
Immune activation rapidly and substantially enhances metabolic outputs (14, 15). Macrophage activation is followed by rapid changes in nutrient flux, which also seems to be necessary for immune activation, indicating that signals produced by immune cells might directly regulate their metabolism. Indeed, studies have highlighted a key role for activated macrophages in controlling energy metabolism and insulin action (15–17). For example, low-grade chronic inflammation is associated with accumulation of macrophages in adipose tissue and predisposition to insulin resistance (15, 18).

In the current study, we aimed to characterize changes in the central carbon metabolic network induced by ERK inhibition and provide a tool to analyze the metabolic flux distribution in macrophages as cross-talk between signal transduction and metabolic events. For this purpose, we used LPS as a model of proinflammatory activation and PD325901 as a selective inhibitor of the MEK/ERK cascade (12). To determine the metabolic state of the cells, we used a tracer-based metabolomics approach with [1,2-13C]2-glucose as the carbon source. Mass isotopomer distribution analysis of key metabolites has been described as a powerful tool to map metabolic flux distribution in several cellular models (19, 20). By tracking the changes in metabolic fluxes induced by ERK signaling modulators, we observed details of the cross-talk between inflammatory signal transduction and metabolic networks. Similar results on glycolytic metabolism were observed in a macrophage cell line in primary cultures of murine peritoneal macrophages and in human monocytes/macrophages.

Materials and Methods

Materials

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). RPMI 1640, FBS, cell culture, and chemical reagents were obtained from Lonza (Cologne, Germany); PD325901, BIRB796, and B78D3 were from CalBiochem (San Diego, CA). [1,2-13C]2-glucose (>99% enriched) was from Isotec (Miamisburg, OH). LPS and reagents for metabolite derivatization were from Sigma-Aldrich (St. Louis, MO). Abs were from Santa Cruz Biotech (Miamisburg, OH). LPS and reagents for metabolite derivatization were from Sigma-Aldrich (St. Louis, MO). Abs were from Santa Cruz Biotech (Santa Cruz, CA), Cell Signaling (Danvers, MA), or Sigma-Aldrich.

Cell culture conditions

RAW 264.7 cells were cultured in RPMI 1640 supplemented with glutamine (2 mM), 10% FBS, and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin) at 37°C in 5% CO2. When cells reached 80% confluence, the medium was replaced with a medium containing only 2% FBS. After overnight serum reduction, cell cultures were loaded with [1,2-13C]2-glucose and treated with 0.5 µM PD325901 and 500 ng/ml LPS for the indicated periods of time. The same procedure was used for studies with p38 and JNK inhibitors but in the absence of labeled glucose. Following incubation, the medium was removed, and cells were scraped off the dishes and processed for RNA, proteins, and intracellular metabolites. Murine peritoneal macrophages and human monocyte/macrophages were prepared (14, 21) and were used as described for the RAW 264.7 cells.

Flow cytometry

Cells were harvested and washed in PBS. After centrifugation at 4°C for 5 min and 1000 x g, cells were resuspended in Annexin V binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2) and labeled with Annexin V-FLICA solution and/or propidium iodide (PI) (100 µg/ml) for 15 min at room temperature in the dark. PI is impermeable to living and early apoptotic cells but stains necrotic and apoptotic dying cells with impaired membrane integrity in contrast to Annexin V, which stains early apoptotic cells.

6-Phosphofructo-2-kinase activity assay

Cells (grown in 6-cm dishes) were homogenized in 1 ml a medium containing 20 mM potassium phosphate ([pH 7.4], 4°C), 1 mM DTT, 50 mM NaF, 0.5 phenylmethylsulfonyl fluoride, 10 µM leupeptin, and 5% poly (ethylene)glycol. After centrifugation in an Eppendorf centrifuge (15 min), poly(ethylene)glycol was added to the supernatant up to 15% (mass/vol) to fully precipitate the 6-phosphofructo-2-kinase (PFK-2). After resuspension of the pellet in the extraction medium, PFK-2 activity was assayed at pH 8.5 with 5 mM MgATP, 5 mM fructose-6-phosphate, and 15 mM glucose-6-phosphate. One unit of PFK-2 activity is the amount of enzyme that catalyzes the formation of 1 pmol Fru-2,6-bisphosphate (Fru-2,6-P2)/min (22).

Metabolite assays

Fru-2,6-P2 was extracted from cells (cultured in 24-well plates) after homogenization in 100 µl 50 mM NaOH, followed by heating at 80°C for 10 min. The metabolite was measured by the activation of the pyrophosphate-dependent 6-phosphofructo-1-kinase (PFK-1) (22). Glucose and lactate were measured enzymatically in the culture medium (23). Glutamine was determined after deamination to glutamate, which was measured enzymatically using the enzyme glutamate dehydrogenase (23). NO release was determined spectrophotometrically by the accumulation of nitrite and nitrate in the medium (phenol red-free), as described before (14).

Preparation of cell extracts

Cells (grown in six-well dishes) were washed twice with ice-cold PBS and homogenized in 0.2 ml buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM 2-ME, 0.1 mM PMSF, and a protease inhibitor mixture (Sigma-Aldrich). The extracts were vortexed for 30 min at 4°C and centrifuged for 10 min at 13,000 x g. The supernatants were stored at −20°C. Protein levels were determined using the Bio-Rad detergent-compatible protein reagent (Richmond, CA). All steps were carried out at 4°C.

Western blot analysis

Samples of cell extracts containing equal amounts of protein (30 µg/lane) were boiled in 250 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 2% 2-ME and separated on 10% SDS-PAGE. The gels were blotted onto a polyvinylidene fluoride membrane (GE Healthcare, Barcelona, Spain) and processed as recommended by the supplier of the Abs against the murine Ags: phospho-ERK1/2 (9101s), phospho-p38 (9211s), phospho-JNK (9215s), NO synthase 2 (NOS-2; sc-7271), cyclooxygenase 2 (COX-2; sc-1999), liver-type–PFK-2 (L–PFK-2) (sc-10096), and β-actin (A-5441). For PFKB3 isoenzyme of PFK-2 (uPFK-2), specific peptides of the isoenzyme were used to generate polyclonal Abs by immunizing rabbits (New Zealand White) with multiple intradermal injections of 300 µg Ag in 1 ml CFA, followed by boosters with 100 µg Ag in IFA. The blots were developed by the ECL protocol (Amersham), and different exposure times were used for each blot with a charged-coupled device camera in a luminescent-image analyzer (Molecular Imager, Bio-Rad) to ensure linearity of the band intensities.

RNA isolation and RT-PCR analysis

One microgram of total RNA, extracted with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions, was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR, following the instructions of the manufacturer (Roche). Real-time PCR was conducted with SYBR Green on a MyQ real-time PCR System (Bio-Rad), using the SYBR Green method. PCR thermocycling parameters (24) were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. All samples were analyzed for 36B4 expression in parallel. Each sample was run in duplicate and was normalized to 36B4. The replicates were then averaged, and fold induction was determined on ΔΔCt-based fold-change calculations. Primer sequences are available on request.

Measurement of reactive oxygen species and NO synthesis

The generation of reactive oxygen species (ROS) was monitored using dichlorofluorescein diacetate (DCFH-DA). Cells were preincubated with 10 µM DCFH-DA for 15 min and fluorescence was measured using a cell cytomter. For fluorometric NO determination, the cell-permeable fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA) was used. Cells were preincubated with 10 µM DAF-2DA for 15 min, and DAF-2DA fluorescence was measured in a cell cytomter.

Metabolite isolation and isotopologue analysis

Glucose, lactate, and glutamite from the incubation medium were purified, derivatized, and analyzed, as previously described (19). Thus, glucose was purified from culture medium using a tandem set of Dowex-1×8/Dowex-50WX8 (Sigma-Aldrich) ion-exchange columns and converted to its aldonitrile pentaacetate derivative. The ion cluster around m/z 328 was
monitored (carbons 1 to 6 of glucose, chemical ionization). Lactate from
the cell culture media was extracted by ethyl acetate after acidification
with HCl. Lactate was derivatized to its propylethoxylate derivative and
the cluster around m/z 198 (carbons 2 to 5 of lactate, chemical ionization)
was monitored. Lactate was derivatized to its propylethoxylate derivative
and measured fluxes for glucose consumption, lactate production, and
inhibited cell viability (52). Also, total 13C enrichment of ribose (Fig.
1C) but did not affect the levels of the chemokines CCL5 (RANTES),
IL-8, and MIP-1α (Fig. 1G), as described before (32). PD325901 impaired
LPS induction of IL-1β and IL-6 mRNA levels (Fig. 1G) but did not affect the levels of the chemokines CCL1-1 and CCL10 (Fig. 1H). Because cell activation might interfere with viability, the percentage of apoptotic cells was
determined by measuring Annexin V and PI staining. PD325901
moderately inhibited cell viability in resting macrophages but
enhanced apoptosis in LPS-activated cells (Fig. 2A). Moreover,
PD325901 decreased cell numbers at 18 h but did not significantly
inflame the percentage of cells gaging at the S, G2, and M phases of
the cell cycle, which was <18% (Fig. 2A). The oxidation of
DCFH-DA and DAF during LPS activation was measured at 18 h.
PD325901 moderately increased the oxidation of both probes but
impaired the large changes that accompany LPS activation (Fig.
2B). An image of cells after 18 h of treatment is shown in Fig. 2C.

To characterize the metabolic changes induced by ERK1/2
inhibition, RAW 264.7 cells were treated with 0.5 μM of PD325901

network structure

The assumed network scheme corresponds to those in Fig. 5. Each solid
arrow indicates a reversible or irreversible reaction step catalyzed by an
enzyme (or transporter) or one block of enzymes. Dashed lines indicate
regulatory connections (product inhibition by glucose-6-phosphate and
activation of pyruvate kinase by fructose-1,6-bisphosphate (Fru-1,6-P2).
Letters correspond to the reaction steps. Reactions steps A-P account for
glycolysis, as well as pentose phosphate pathway (PPP) and tricarbox-
yclic acid (TCA) cycle enzyme-catalyzed reactions. Some reactions were
neglected and grouped into blocks (e.g., reaction step F representing the
block from GAPDH to pyruvate kinase), and others are assumed to be
involved in rapid equilibriums (e.g., glucose-6-phosphate isomerase).

Metabolites are combined into pools: a first pool for hexose-phosphates,
including glucose-6-phosphate and fructose-6-phosphate; a second pool for
pentose-phosphates accounting for ribose-5-phosphate, ribulose-5-phos-
phate, and xylulose-5-phosphate; and a third pool for oxaloacetate (Oaa)
and malate (Mal). The rest of the metabolic intermediaries are Fru-1,6-P2,
dihydroxyacetonephosphate, GAPDH, sedoheptulose-7-phosphate, erythrose-
4-phosphate, pyruvate (Pyr), acetyl-CoA, citrate, 2-oxoglutarate, and succinyl-
CoA. Reaction steps A, D, T; U, X, Y, and Z represent the inputs and outputs
of the metabolic system.

Estimation of flux dependencies on enzyme activities

Estimation of flux dependencies on enzyme activities are based on the
identification of control coefficients with fixed signs. The sign and magni-
itude of control coefficients depend on the topology of the network, the
stoichiometry of the reactions, and the magnitudes of fluxes and of regu-
ulatory dependencies (enzyme–substrate affinity, inhibitions, and activa-
tions) (23). Magnitudes of regulatory dependencies are unknown, but the
sign of some control coefficients are fixed, irrespective of these magni-
tudes. Others are sign indeterminate, meaning that they can be positive and
negative, and some are always zero.

Results

Characterization of macrophage activation after ERK1/2
inhibition

To characterize the response of RAW 264.7 cells to the MEK/ERK
selective inhibitor PD325901 (12) and LPS activation, several
functional markers were used. Fig. 1A shows the dose-dependent
inhibition of ERK1/2 phosphorylation by PD325901 in LPS-acti-
vated cells. The inhibitor significantly decreased LPS-induced
NOS-2 and COX-2 protein levels (Fig. 1B), as well as nitrite
plus nitrate accumulation in the medium (Fig. 1C). At the meta-
bolic level, PD325901 decreased the basal levels of Fru-2,6-P2,
a potent activator of the glycolytic flux, and impaired its increase
induced by LPS (Fig. 1D). This was associated with a decrease in the
expression of the highly active uPFK-2 isoform induced by LPS and,
concomitantly, a reduction in total PFK-2/fructose-2,6-
bisphosphatase (FBPase-2) activity (Fig. 1E). Similar results in terms of ERK inhibition, NOS-2 and COX-2 expression, and
changes in PFK-2 isoenzymes were observed with the MEK/ERK
inhibitors SL327 and PD98059 (data not shown). Changes in
mRNA correlated with those observed for protein levels of
NOS-2, COX-2, uPFK-2, and L–PFK-2 (Fig. 1F). Moreover, to reinforce the specific effect of ERK1/2 inhibition on LPS activa-
tion, an increase in IL-12p40 (IL-12) and decrease in TNF-α
mRNA levels were observed (Fig. 1G), as described before (32).
PD325901 impaired LPS induction of IL-1β and IL-6 mRNA
levels (Fig. 1G) but did not affect the levels of the chemokines
CXCL-1 and CXCL-10 (Fig. 1H). Because cell activation might
interfere with viability, the percentage of apoptotic cells was
determined by measuring Annexin V and PI staining. PD325901
moderately inhibited cell viability in resting macrophages but
enhanced apoptosis in LPS-activated cells (Fig. 2A). Moreover,
PD325901 decreased cell numbers at 18 h but did not significantly
inflame the percentage of cells gaging at the S, G2, and M phases of
the cell cycle, which was <18% (Fig. 2A). The oxidation of
DCFH-DA and DAF during LPS activation was measured at 18 h.
PD325901 moderately increased the oxidation of both probes but
impaired the large changes that accompany LPS activation (Fig.
2B). An image of cells after 18 h of treatment is shown in Fig. 2C.

To characterize the metabolic changes induced by ERK1/2
inhibition, RAW 264.7 cells were treated with 0.5 μM of PD325901

Statistical analysis

The data shown are the means ± SD of three or four experiments. Sta-
tistical significance was estimated with the Student t test for unpaired
observations. Significance of isotopologue data (Table I) was analyzed
using two-way ANOVA.

Gas chromatography/mass spectrometry

Mass spectral data were obtained on a QP2010 mass selective detector
connected to a GC-2010 gas chromatograph (Shimadzu Scientific Instru-
mements, Kyoto, Japan) using a 30-m length, 250 μm film thickness, and
with HCl. Lactate was derivatized to its propylethoxylate derivative
and analyzed by three inde-
pendent automated injections of 1 μl each sample and were accepted only if
the standard sample deviation was <1% of the normalized peak intensity.

Estimation of internal fluxes based on the measured 13C
redistribution

Each 13C-labeled metabolite corresponds to a different isotopomer, which
differs only in the labeling state of its individual atoms (26). For a specific
metabolite, the number of possible isotopomers, 2n, depends on the
number, n, of carbons for each metabolite. The relative abundance of
product isotopomers depends on the labeled status of the substrates (50%-
1,2-[13C]glucose) and the flux distribution throughout the metabolic
network (27–29). Isotopomer abundances can be predicted by solving
a system of isotopomer mass balance equations, where each equation
describes the dependency of each isotopomer abundance on fluxes and
isotopomer abundance of other metabolites (30). The space of solution for
each condition (vehicle, LPS, PD325901, and PD325901+LPS) is scanned
by solving the system of equations for feasible combinations of flux values
for all reaction steps. All combinations satisfied the constraints associated
with network topology described below, stoichiometry for each reaction,
and predicted fluxes for glucose consumption, lactate production, and
glutamine consumption (23). Also, total [13C] enrichment of ribose (2
molecules with one [13C]) and [2
m (2 molecules with two [13C]) were measured for each condition (vehicle,
LPS, and PD325901+LPS).

Network structure

The assumed network scheme corresponds to those in Fig. 5. Each solid
arrow indicates a reversible or irreversible reaction step catalyzed by an
enzyme (or transporter) or one block of enzymes. Dashed lines indicate
regulatory connections (product inhibition by glucose-6-phosphate and
activation of pyruvate kinase by fructose-1,6-bisphosphate (Fru-1,6-P2).
Letters correspond to the reaction steps. Reactions steps A–P account for
glycolysis, as well as pentose phosphate pathway (PPP) and tricarbox-
ylic acid (TCA) cycle enzyme-catalyzed reactions. Some reactions were
neglected and grouped into blocks (e.g., reaction step F representing the
block from GAPDH to pyruvate kinase), and others are assumed to be
involved in rapid equilibriums (e.g., glucose-6-phosphate isomerase).
and/or 500 ng/ml of LPS. Glucose and glutamine consumptions and lactate production after 1, 4, and 8 h of incubation are presented in Fig. 3A. Both glucose consumption and lactate production were lower in the presence of PD325901, whereas glutamine consumption was not affected. LPS stimulation increased glucose consumption and lactate production but did not induce these effects in the presence of PD325901. Interestingly, LPS increased glutamine consumption, regardless of the presence of PD325901. The ratios between lactate production and glucose consumption, as well as glucose/glutamine consumption are shown (Fig. 3B). Because glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activities might be affected by PD325901 in LPS-activated cells, the time course of their activity was measured, with a modest transient increase at 8 h, independent of PD325901 treatment (Fig. 3C). In addition to RAW 264.7 cells, the effect of the inhibition of ERK on LPS-dependent activation of glycolysis was investigated in peritoneal murine macrophages and in human monocyte/macrophages. As Fig. 3D shows, LPS challenge promoted uPKF-2 expression and an increase in Fru-2,6-P₂ levels in these macrophages. Treatment with PD325901 blunted the effect of LPS on both uPKF-2 expression and Fru-2,6-P₂ increase. In addition to this, good correlations between uPKF-2/Fru-2,6-P₂ levels and glucose consumption and lactate production were observed in the three types of macrophages analyzed (Fig. 3E).

Inhibition of p38 and JNK MAPKs with selective inhibitors was also evaluated in RAW 264.7 cells. The p38 inhibitor BI-RB796 did not significantly affect cell viability at 0.5 μM (Fig. 4A; previous p38 inhibitors exhibited cytotoxic effects) and suppressed p38 phosphorylation (Fig. 4B). However, the selective JNK inhibitor B178D3 significantly decreased cell viability at the minimal concentration required to suppress JNK phosphorylation in response to LPS (Fig. 4A, 4B); p38 inhibition did not influence the LPS-dependent uPKF-2 expression (Fig. 4C), the increase in Fru-2,6-P₂ levels, or the glycolytic flux in RAW 264.7 cells (Fig. 4D). With regard to JNK inhibition, it is difficult to draw conclusions about the effects on cell viability. Although treatment with B178D3 decreased uPKF-2 levels at 8 h after LPS treatment (Fig. 4C); the Fru-2,6-P₂ levels at 8 h were 81% of those of LPS (Fig. 4D), which contrasts with the 69% inhibition observed after MEK/ERK inhibition (Fig. 1D).

**Measuring isotopologue distribution**

The metabolism of [1,2-¹³C₂]-D-glucose causes rearrangement, exchange, or loss of the [¹³C] label, which is incorporated into the glucose metabolic intermediates in specific patterns. The [¹³C] label enrichment of these intermediates also depends on the dilution of their unlabeled counterparts. Thus, a specific isotopologue distribution provides information on the flux of metabolites along the forward and reverse pathways of substrate cycles. RAW 264.7 cells treated with 0.5 μM of PD325901 and/or 500 ng/ml of LPS were incubated for 18 h with 10 mM glucose 50% enriched in [1,2-¹³C₂]-d-glucose, and the isotopologue distributions were measured (Table I).
Glucose and lactate in the medium. Glucose enrichment was not significantly affected either by PD325901 or LPS treatment alone or in combination (data not shown), indicating that the macrophages did not release newly synthesized glucose into the medium. With regard to lactate, \(^{13}\)C incorporation through glycolysis results in the formation of lactate with two \(^{13}\)C (m2 lactate). m1 lactate mainly originates from the decarboxylation of \([1,2-{^{13}}\text{C}]\) glucose through the oxidative branch of the PPP and its subsequent recycling to glycolysis through the nonoxidative branch of PPP or by the action of Pyr cycling (mediated by phosphoenolpyruvate carboxykinase or malic enzyme). The parameter PPC (PPC = \([m1/m2]/[3+(m1/m2)]\)) that represents the contribution of these last two pathways to lactate formation.

Ribose in RNA. Pentose phosphates can be synthesized from glucose or glycolytic intermediates through two pathways: the oxidative and nonoxidative branches of the PPP. The ratio of m1/m2 among the different ribose isotopologue fractions represents the contribution of the oxidative versus the nonoxidative branch of PPP. This ratio changes from 1.29 in control to 1.10 in the presence of PD325901, 1.08 after LPS activation, and 1.13 in the presence of both, indicating a similar decrease in the oxidative branch of ribose synthesis in all cases. A part of RNA ribose was not synthesized de novo, because the nonlabeled nucleotides that existed before the incubation were reused in subsequent generations. This reused part contributed to the value of the nonlabeled fraction (m0) of defined RNA ribose. The lower m0 value found in control and LPS conditions suggested that PD325901 addition resulted in diminishing de novo synthesis of nucleotides.

Glutamate in the medium. Label distribution in glutamate allows us to estimate the relative contributions of pyruvate carboxylase and pyruvate dehydrogenase (PDH) to the TCA cycle (19). The fact that glutamate was mainly labeled at the fourth and fifth positions in all incubation conditions demonstrated that \([1^{\text{C}}\text{C}]\) glucose entered the TCA cycle, mainly by PDH in RAW 264.7 cells, regardless of treatment. Furthermore, glutamate labeling increased in the presence of PD325901 and/or LPS, indicating that both stimuli and their combination increased the exchange between glutamate and \(\alpha\)-ketoglutarate.

Estimation of internal fluxes

Mass isotopomer distribution analysis was completed with a numerical estimation of internal fluxes. To reveal the profiles of internal metabolic fluxes that underlie the isotopologue distributions corresponding to ERK1/2 inhibition in resting or activated cells, we analyzed the label distributions using the approach described in Materials and Methods. The metabolic network ana-
The incubation of RAW 264.7 cells with 2.5% of the Pyr produced from glucose enters the TCA cycle in and glutamine are mainly rerouted to lactate, and only phosphate pool to Pyr (flux through F), suggesting that glucose after incubation with PD325901 (regulated by signal transduction throughout MEK/ERK rows represent the proposed activities that are regulated is depicted in Fig. 5, and the resulting numerical estimation of fluxes throughout the main steps in the metabolic network is presented in Fig. 6. The flux profile results indicated that RAW 264.7 cells under basal conditions were mainly glycolytic, having most of the consumed glucose (flux through A) converted into lactate (flux through T). The consumed glutamine in the TCA cycle (flux through U) was transformed to Oaa-Mal (fluxes through O and P), mainly recycled to Pyr (flux through R), and excreted into the medium as lactate. Flux through PDH (flux through L) was ~40–80 times lower than that from the triose phosphate pool to Pyr (flux through F), suggesting that glucose and glutamine are mainly rerouted to lactate, and only ~1.25–2.5% of the Pyr produced from glucose enters the TCA cycle in RAW 264.7 cells. The incubation of RAW 264.7 cells with PD325901 produced a clear decrease in almost all the analyzed fluxes. Furthermore, LPS increased the glycolytic flux, although this was inhibited by PD325901. With regard to PPP fluxes, observed differences in fluxes through B, G, H, and I showed a clear decrease in the presence of PD325901. A smaller decrease in the PPP fluxes was induced by LPS and when cells were coincubated with PD325901 and LPS. These differences in flux profiles are consistent with the different consumptions and productions of glucose, lactate, and glutamine and de novo synthesis of nucleotides.

**Flux dependencies on enzyme activities**

At a specific network description of central carbon metabolism with a particular topology, reaction stoichiometry, flux values and sign

<table>
<thead>
<tr>
<th>Metabolite Distribution</th>
<th>Vehicle</th>
<th>LPS</th>
<th>PD325901</th>
<th>PD325901+LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate C1–C3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m0</td>
<td>0.783 ± 0.0033</td>
<td>0.783 ± 0.0116</td>
<td>0.790 ± 0.004</td>
<td>0.772 ± 0.006*</td>
</tr>
<tr>
<td>m1</td>
<td>0.0200 ± 0.0033</td>
<td>0.0156 ± 0.0026**</td>
<td>0.0184 ± 0.0016</td>
<td>0.0173 ± 0.0020</td>
</tr>
<tr>
<td>m2</td>
<td>0.198 ± 0.004</td>
<td>0.212 ± 0.006**</td>
<td>0.190 ± 0.004**</td>
<td>0.211 ± 0.004*</td>
</tr>
<tr>
<td>PPC</td>
<td>0.033 ± 0.006</td>
<td>0.024 ± 0.004**</td>
<td>0.031 ± 0.003</td>
<td>0.027 ± 0.003*</td>
</tr>
<tr>
<td>Ribose C1–C5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m0</td>
<td>0.752 ± 0.006</td>
<td>0.766 ± 0.002</td>
<td>0.801 ± 0.005**</td>
<td>0.771 ± 0.003*</td>
</tr>
<tr>
<td>m1</td>
<td>0.121 ± 0.004</td>
<td>0.103 ± 0.003*</td>
<td>0.092 ± 0.004**</td>
<td>0.102 ± 0.001**</td>
</tr>
<tr>
<td>m2</td>
<td>0.0938 ± 0.0026</td>
<td>0.0954 ± 0.0012</td>
<td>0.0840 ± 0.0012*</td>
<td>0.0905 ± 0.0017</td>
</tr>
<tr>
<td>m3</td>
<td>0.0206 ± 0.0015</td>
<td>0.0212 ± 0.0006</td>
<td>0.010 ± 0.0086*</td>
<td>0.0234 ± 0.0019</td>
</tr>
<tr>
<td>m1/m2</td>
<td>1.29 ± 0.00</td>
<td>1.08 ± 0.05**</td>
<td>1.10 ± 0.06**</td>
<td>1.13 ± 0.02**</td>
</tr>
<tr>
<td>Glutamate C2–C5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m0</td>
<td>0.974 ± 0.001</td>
<td>0.959 ± 0.002**</td>
<td>0.960 ± 0.003**</td>
<td>0.956 ± 0.002**</td>
</tr>
<tr>
<td>m1</td>
<td>0.0050 ± 0.0006</td>
<td>0.0111 ± 0.0008**</td>
<td>0.0079 ± 0.0020**</td>
<td>0.0099 ± 0.0008**</td>
</tr>
<tr>
<td>m2</td>
<td>0.0201 ± 0.0005</td>
<td>0.0287 ± 0.0009**</td>
<td>0.0308 ± 0.0012**</td>
<td>0.033 ± 0.006**</td>
</tr>
<tr>
<td>Glutamate C2–C4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m0</td>
<td>0.975 ± 0.001</td>
<td>0.960 ± 0.001**</td>
<td>0.960 ± 0.003**</td>
<td>0.956 ± 0.001**</td>
</tr>
<tr>
<td>m1</td>
<td>0.0245 ± 0.0007</td>
<td>0.0390 ± 0.0013**</td>
<td>0.0390 ± 0.0026**</td>
<td>0.0435 ± 0.0015**</td>
</tr>
<tr>
<td>m2</td>
<td>0.0007 ± 0.0003</td>
<td>0.0011 ± 0.0005</td>
<td>0.0007 ± 0.0012</td>
<td>0.0006 ± 0.0006</td>
</tr>
<tr>
<td>Contributions to TCA cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.01 ± 0.04*</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>PDH</td>
<td>0.96 ± 0.01</td>
<td>0.96 ± 0.02</td>
<td>0.99 ± 0.04*</td>
<td>0.98 ± 0.02</td>
</tr>
</tbody>
</table>

Isotopologue distribution of lactate (fragment C1–C3) and glutamate (fragments C2–C5 and C2–C4) secreted into the culture medium and RNA ribose (fragment C1–C5) after 18 h without LPS or PD325901 (vehicle) or with LPS and PD325901 individually or in combination. PPC parameter was estimated from the formula (m1/m2/3 + m1/m2) using lactate isotopologue fractions. Pyruvate carboxylase and PDH contributions to TCA cycle were estimated using m2C2–C4/m2C2–C5 and (m2C2–C5 estimated from the formula (m1/m2)/{3 + (m1/m2)} using lactate isotopologue fractions. Pyruvate carboxylase and PDH contributions to TCA cycle were

**FIGURE 5.** Cross-talk between MEK/ERK and key aspects of macrophage metabolism. Gray arrows represent the proposed activities that are regulated by signal transduction throughout MEK/ERK after incubation with PD325901 (right panel) or LPS (left panel). Positive (+) or negative (−) symbols predict activation or inhibition, respectively. ACoA, acetyl-CoA; Cit, citrate; DHAP, dihydroxyacetonephosphate; E4P, erythrose-4-phosphate; GAP, fru-1,6P₂, glyceraldehyde-3-phosphate; HexP, hexose phosphates; αKG, 2-oxoglutarate; PenP, pentose phosphates; S7P, sedoheptulose-7-phosphate; Succ, succinyl-CoA.
of the regulatory dependencies (positive for enzyme-substrate dependencies and activations, and negative for inhibitions), dependencies among specific activities and the flux through a specific reaction depend on the relative magnitudes of the regulatory dependencies, which are unknown. However, some of these dependencies can be mainly positive or negative (23). A positive dependency indicates that a change in the enzyme activity is compatible or predicts a change in the flux that follows the same direction, irrespective of the magnitude of the regulatory dependencies. This means that an increase in the activity will induce an increase in the flux, whereas decreasing the activity will also decrease the flux. In contrast, a negative dependency indicates that changes in the activity will induce an inverse effect on the changes in the flux. Fig. 7A shows some of these sign-fixed dependencies for the main glycolytic and PPP fluxes with respect to changes in the activities of glucose uptake + hexokinase (reaction step A in Fig. 5), PFK-1 (reaction step C in Fig. 5), lactate dehydrogenase + lactate exchange (reaction step T in Fig. 5), PDH (reaction step L in Fig. 5), and G6PDH+6PGDH (reaction step B in Fig. 5).

The analysis of the compatibility of the measured changes in enzyme activities in the context of topology, stoichiometry, fluxes, and regulations affecting the central carbon metabolism provides fundamental information for interpreting the effects of LPS stimulation and PD325901 inhibition. Changes in glycolytic activity by regulating PFK-1 activity (reaction step C) are expected as a consequence of the changes in basal levels of Fru-2,6-P2 (Fig. 1D), which is a potent activator of the glycolytic flux. More modest changes in G6PDH and 6PGDH activities (reaction step B) were recorded (Fig. 3C). Fig. 7B shows the compatibility of the direction of these changes in enzyme activities and the direction of changes in fluxes. In cells treated with PD325901, the decrease in PFK-1 (reaction step C) activity alone explains the decrease in the glycolytic fluxes (reaction steps A, C, F, T, and L) but not the changes in PPP fluxes (reaction steps B, G, H, and I). In contrast, a decrease in the activities of G6PDH+6PGDH (reaction step B) alone explains the observed changes in PPP fluxes but not all of the changes observed in glycolytic fluxes. Interestingly, this showed that changes in PFK-1 and G6PDH+6PGDH occur simultaneously, as has been experimentally observed, and could explain the changes in both glycolytic and PPP fluxes. In cells treated with LPS, the strong PFK-1 activation that follows the high levels of Fru-2,6-P2 observed could qualitatively explain all of the changes in glycolytic and PPP fluxes. An increase in the activities of G6PDH+6PGDH alone will result in an increase in PPP fluxes, but this was not observed, given that the high levels of Fru-2,6-P2 favored PFK-1 activation in the resulting flux profile. When cells were treated simultaneously with PD325901 and LPS, the slight increase in Fru-2,6-P2 was not sufficient to activate the glycolytic flux profile characteristic of PFK-1 activation. The slight decrease in the PPP fluxes observed can be explained by the combined effect of changes in both PFK-1 and G6PDH+6PGDH activities.

**Discussion**

A detailed [1,2-13C]glucose tracer-based metabolomics approach, together with measured changes in glucose and glutamine consumption and lactate production, was used to characterize the effects of MEK/ERK inhibition on the basic metabolic response to LPS stimulation in macrophages. One of our previous studies showed that classic versus alternative macrophage activation involved the expression of specific sets of metabolic enzymes intended to cope with the energy demands of the activated cells (14). However, the finding that a single hit (i.e., MEK inhibition) might influence the LPS response in metabolic terms offers a new view on the cross-talk between cell activation and basic energy metabolism. Moreover, these effects on MEK/ERK inhibition were also observed in cultured peritoneal macrophages and in human monocytes differentiated to macrophages (21, 24). From a bioenergetics point of view, macrophages are essentially glycolytic cells (16, 33, 34) using anaerobic glycolysis to metabolize glucose. One of the regulators of glucose metabolism in macrophages is the increase in Fru-2,6-P2 levels, which activates the flux through...
PFK-1 (14, 35). In many glycolytic cells, Fru-2,6-P$_2$ levels are tightly regulated through balancing PFK-2/FBPase-2 activities. Four genes encode the PFK-2/FBPase-2 in mammals. The L-type is encoded by the $PFKB1$ gene and is mainly expressed in the liver and muscle. The uPFK-2 is encoded by the $PFKB3$ gene and has a predominantly kinase activity, with lower bisphosphatase activity. This gene is induced by hypoxia and regulated by phosphorylation, playing a role in the high glycolytic rate of various cell types, such as cancer cells (35, 36). In macrophages, innate and classic activation, but not the alternative IL-4/IL-13 stimulation, switches the expression of the PFK-2/FBPase-2 isofrom in $PFKB1$ prevailing in resting cells to $PFKB3$, resulting in an increase in Fru-2,6-P$_2$ levels and glycolytic flux (14). Interestingly, MEK/ERK inhibition impaired the LPS-dependent expression of uPFK-2, thus decreasing Fru-2,6-P$_2$ levels, PFK-2 activity, and, as expected, glucose consumption and lactate production but without changes in glutamine/glutamate consumption. The ability of the MEK/ERK pathway to prevent the switch from L–PFK-2 to uPFK-2 in response to LPS was unexpected and revealed fine tuning of macrophage activation. Other changes induced by LPS, such as a decrease in PPP fluxes, were not affected by PD325901. Indeed, using the same approach, a selective p38 inhibitor (12) did not interfere with the LPS enhancement of glycolytic flux, including the increase in uPFK-2/Fru-2,6-P$_2$ levels. However, the lack of a JNK inhibitor preserving cell viability complicates this study in these cells. Even though, analysis of lactate release and uPFK-2/Fru-2,6-P$_2$ levels in cells treated with BI78D3 and activated with LPS suggests a minor (if any) effect of JNK inhibition on carbon metabolism in RAW 264.7 cells.

The cross-talk between MEK/ERK and central carbon metabolism is summarized in Fig. 5. From an analytical point of view, macrophage activation with LPS is characterized by enhanced flux through PFK-1, via a Fru-2,6-P$_2$-increase, and explains the increases in the glycolytic pathway and the decrease in the reactions in the PPP. However, the transient (peak at 8h), but statistically significant, increase in activity through the G6PDH+6PGDH block should lead to changes in the opposite direction, which are likely to mediate the decrease in fluxes throughout the PPP via increased PFK-1 activity. Interestingly, the flux profile changing following PD325901 inhibition, with or without LPS, and could not be explained by the change in PFK-1 alone. Changes in both PFK-1 and in G6PDH+6PGDH are required to explain the observed flux profile. Indeed, an additional regulator of the cross-talk at the Fru-2,6-P$_2$ level is the expression of TIGAR, a p53-inducible enzyme profile. Indeed, an additional regulator of the cross-talk at the Fru-2,6-P$_2$ level is the expression of TIGAR, a p53-inducible enzyme.

Acknowledgments

We thank Verónica Terrón for technical help.

Disclosures

The authors have no financial conflicts of interest.

References


