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

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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 [1,2-13C]2-glucose tracer-based metabolomic approach was used to examine the metabolic flux distribution in these cells after MEK/ERK inhibition. Bioinformatic tools were used to analyze changes in mass isotopomer 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: 1402–1410.

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 become life-threatening, as observed in patients with severe sepsis or septic shock. Therefore, immune responses must be tightly regulated (3, 5, 6).

NF-κB 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).

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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-^{13}C]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, BIBR796, and BI78D3 were from Calbiochem (San Diego, CA). [1,2-^{13}C]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 Biotechnology (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% subconfluency, the medium was replaced with a medium containing only 2% FBS. After overnight serum reduction, cell cultures were loaded with [1,2-^{13}C]2-glucose and treated with 0.5 μM PD325901 and 50 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 scrapped off the dishes and processed for RNA, proteins, and intracellular metabolites. Murine peritoneal macrophages and human monocytes/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 × 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-PE 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 EDTA, 50 mM NaF, 0.5 mM phenylmethanesulfonyl 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 Fructose-6,2-bisphosphate (Fructose-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 30 min. The metabolite was measured by the activation of the pyrophosphate-dependent 6-phosphofructo-2-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), 1.5 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 × 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 in 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-IκB (9251s), NO synthase 2 (NOS-2; sc-72777), cyclooxygenase 2 (COX-2; sc-9997), liver-type–PFK-2 (L–PFK-2) (sc-10096), and β-actin (A-5494). For PFKB3 isoform of PFK-2 (αPFK-2), specific peptides of the isoenzyme were used to generate monoclonal 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 inIFA. The Abs were preincubated with 10 μg/ml PD325901 and incubated for 15 min at 4°C. Protein levels were determined using the Bio-Rad detergent-compatible protein reagent (Richmond, CA). All steps were carried out at 4°C.

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 MyiQ 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 ∆ΔCt-based fold-change calculations. Primer sequences are available on request.

Measurement of reactive oxygen species and NO formation

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 cytometer. 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 cytometer.

Metabolite isolation and isotopologue analysis

Glucose, lactate, and glutamate 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 propylamide-heptafluorobutyric form, and the cluster around m/z 328 (carbons 1 to 3 of lactate, chemical ionization) was monitored. Glutamate was separated from the medium using ion-exchange chromatography and converted to its n-trifluoroacetyl-n-butyryl derivative. The ion clusters around m/z 198 (carbons 2 to 5 of glutamate, electron impact ionization) and m/z 152 (carbons 2 to 4 of glutamic acid) (EI cycle electron ionization) were monitored. RNA ribose was purified, derivatized, and analyzed, as previously described (20). In detail, RNA ribose was isolated by acid hydrolysis of cellular RNA after TRizol purification of cell extracts. Ribose isolated from RNA was derivatized to its aldonolactone acetate form using hydroxylamine in pyridine and acetic anhydride, and the ion cluster around the m/z 256 (carbons 1 to 5 of ribose, chemical ionization) was monitored. Spectral data were corrected using regression analysis to extract natural [13C] enrichment from the results (25). Measurement of [13C] label distribution determined the different relative distribution percentages of isotopologues, and m0 (molecules without any [13C] labels), m1 (molecules with one [13C]), m2 (with two [13C]), and so forth were reported as molar fractions.

Gas chromatograms/mass spectrometry

Mass spectral data were obtained on a QP2010 mass selective detector connected to a GC-2010 gas chromatograph (Shimadzu Scientific Instruments, Kyoto, Japan) with a 30-m TRebal Rtx-51 column (Agilent, Santa Clara, CA). The GC oven was maintained at 80°C for 4 min, ramped at 5°C/min to 150°C, and held for 10 min. The transfer line, 250°C, and mass chromatography source, 200°C. A Varian VF-5 capillary column (30 m in length, 25 µm in diameter, and with a 0.25-µm film thickness) was used to analyze all of the compounds. In vitro experiments were carried out using duplicate cultures each time for each treatment regimen. Mass spectral analyses were carried out by three independent 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 measured fluxes for glucose consumption, lactate production, and [13C] enrichment of ribose (23). Also, total [13C] enrichment of ribose (\(\Sigma m = m1 + m2 + m3\)) was applied to fix the differential de novo RNA synthesis (step D in Fig. 5) among the analyzed conditions (Table I). For reversible reactions, exchange fluxes, which account for the cycle through the forward and backward reactions (29, 31), were considered in addition to the net reactions. Ratios m1/(m1+m2) and m2/(m1+m2) for lactate and glutamate C2–C4 and C2–C5 fragments and m1/(m1+m2+m3), m2/(m1+m2+m3), and m3/(m1+m2+m3) for ribose measured experimentally (Table I) and predicted for different flux distributions were compared (least squares). The 20 combinations of flux distributions with a best fitting were taken for each case (vehicle, PD325901, 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. Reaction steps A–P account for all metabolic level, PD325901 decreased the basal levels of Fru-2,6-P2, plus nitrate accumulation in the medium (Fig. 1C). At the metabolic 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 activation, 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 influenced 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 affect the percentage of cells gating 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
The metabolism of [1,2-\textsuperscript{13}C\textsubscript{2}]glucose causes rearrangement, exchange, or loss of the [\textsuperscript{13}C] label, which is incorporated into the glucose metabolic intermediates in specific patterns. The [\textsuperscript{13}C] label enrichment of these intermediates also depends on the dilution or loss of the [\textsuperscript{13}C] label. 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 were incubated for 18 h with 10 mM glucose 50% enriched in [\textsuperscript{1,2-13}C\textsubscript{2}]-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, [13C] incorporation through glycolysis results in the formation of lactate with two [13C] (m2 lactate). m1 lactate mainly originates from the decarboxylation of [13C] caused by the metabolism of [1,2-[13C]2]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 over glycolysis was determined at 8 h. Results show the mean ± SD of three experiments. *p < 0.05, **p < 0.01 versus the untreated condition; #p < 0.05 versus no MEK inhibitor.

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 [13C] from [1,2-[13C]2]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 α-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-
lyzed 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 R), and TCA cycle (flux through U) was transformed to Oaa-Mal (fluxes through R), and the consumed glutamine in the TCA cycle (flux through U) was transformed to Oaa-Mal (fluxes through R), and lactate (flux through T). The consumed glutamine in the TCA cycle (flux through U) was transformed to Oaa-Mal (fluxes through R), and lactate (flux through T).

Table I. Isotopologue distribution in different metabolites

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

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 \((m_1/m_2) - (m_0/3 + (m_1/m_2))\) using lactate isotopologue fractions. Pyruvate carboxylase and PDH contributions to TCA cycle were estimated using \(m_2C_2–C_4/m_2C_2–C_5\) and \((m_2C_2–C_5/m_2C_2–C_4)\), respectively. Values are expressed as mean ± SD.

*p < 0.05, **p < 0.01 versus the untreated condition; #p < 0.05, ##p < 0.01 versus no PD325901.

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. AcCoA, acetoyl-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; SuccC, 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]2]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...
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 isoform from PFKB1 prevailing in resting cells to PFKB3, resulting in an increase in Fru-2,6-P2 levels and glycolytic flux (14). Interestingly, MEK/ERK inhibition impaired the LPS-dependent expression of uPFK-2, thus decreasing Fru-2,6-P2 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-P2 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-P2 levels in cells treated with B178D3 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-P2 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 changed 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-P2 level is the expression of TIGAR, a p53-inducible enzyme that hydrolyzes Fru-2,6-P2 to fructose-6-phosphate (37, 38). We investigated whether TIGAR was regulated by p53 levels in macrophages. However, p53 was only upregulated at the end of the activation process (data not shown), when there was a large increase in the synthesis of ROS and reactive nitrogen species. Interestingly, MEK/ERK inhibition decreased ROS production by LPS-activated macrophages, conferring an interference of this MAPK on the LPS-dependent activation program of the macrophage. However, at the same time, MEK/ERK inhibition moderately enhanced (8 h) or maintained (18 h) the metabolic flux through the G6PDH pathway, excluding a sequential dependence of these pathways during activation. In agreement with these results, p65Hcific-deficient mice, which exhibit an attenuated ROS synthesis due to a defect in the activation of the NADPH oxidase complex, also exhibit a marked reduction in ERK activation (39).

Finally, ERK1/2 activation in macrophages under proinflammatory conditions has been associated with different pathophysiological situations, ranging from cancer to insulin resistance. For example, macrophage infiltration increases during tumor progression in mouse models of lung cancer, but the combined inhibition of MEK and PI3K ablated macrophage-mediated increases in epithelial growth, enhancing animal survival (40); in contrast, it was shown that the proinflammatory cytokine IL-1β reduces insulin receptor substrate 1 expression and prevents Akt activation, leading to insulin resistance through a mechanism that is partly mediated by ERK activation (41–43). Therefore, ERK1/2 regulation appears to be an important mediator of macrophage function. In summary, the presented quantitative analysis revealed many more details about the metabolic effects of the signaling regulators studied, showing that the exploration of metabolic effects provides important details that cannot be shown by only qualitative analysis of experimental data. Our work is an example of quantitative analysis of the cross-talk between signal transduction and metabolism in RAW 264.7 cells.

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References
1. Gordon, S., and F. O. Martinez. 2010. Alternative activation of macrophages: mechanism and functions. Immunity 32: 593–604.
2. Gordon, S. 2007. The macrophage: past, present and future. Eur. J. Immunol. 37 (Suppl. 1): 89–817.
3. Nathani, C. 2002. Points of control in inflammation. Nature 420: 846–852.
4. Hu, X., S. D. Chakravarty, and L. B. Ivashkiv. 2008. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. Immunol. Rev. 226: 41–56.
5. Martínez, F. O., A. Sica, A. Mantovani, and M. Locati. 2008. Macrophage activation and polarization. Front. Biosci. 13: 453–461.
6. Pasare, C., and R. Medzhitov. 2004. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect. 6: 1382–1387.
7. Martinez, F. O., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25: 677–686.
8. Nathan, C., and A. Ding. 2010. Nonresolving inflammation. Cell 140: 871–882.
9. Martinez, F. O., S. Gordon, M. Locati, and A. Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J. Immunol. 177: 7303–7311.
10. Rao, K. M. 2001. MAP kinase activation in macrophages. J. Leukoc. Biol. 69: 3–10.
11. Rao, K. M., T. Meighhan, and L. Bowman. 2002. Role of mitogen-activated protein kinase activation in the production of inflammatory mediators: differences between primary rat alveolar macrophages and macrophage cell lines. J. Toxicol. Environ. Health A 65: 757–768.
12. Bain, J., L. Plater, M. Elliott, N. Shpiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alexis, and P. Cohen. 2007. The selectivity of protein kinase inhibitors: a further update. Biochem. J. 408: 297–315.
13. Steibbins, J. L., S. K. De, T. Machleidt, B. Becattini, J. Vazquez, C. Kunzten, L. H. Chen, J. F. Cellitti, M. Riel-Mehan, et al. 2008. Identification of a new JNK inhibitor targeting the JNK-JIP interaction site. Proc. Natl. Acad. Sci. USA 105: 16809–16813.
14. Rodríguez-Prados, J. C., P. G. Traves, J. Cuenca, D. Rico, J. Aragones, P. Martin-Sanz, M. Cascante, and L. Bosca. 2010. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J. Immunol. 185: 605–614.
15. Odegaard, J. I., R. R. Ricardo-Gonzalez, A. Red Eagle, D. Vats, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alexis, and P. Cohen. 2007. The selectivity of protein kinase inhibitors: a further update. Biochem. J. 408: 297–315.
16. Wuyts, W. S., B. J. Jansen, and F. E. M. M. Van De Wiel. 2001. Binding properties of the JAK/STAT system. J. Mol. Biol. 308: 545–561.
17. Bhattacharyya, N., P. Mascola, A. Sica, C. J. Hastie, J. A. Meighigan, and L. Plater. 2007. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25: 453–461.
18. Pasare, C., and R. Medzhitov. 2004. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect. 5: 1382–1387.
19. Martinez, F. O., S. Gordon, M. Locati, and A. Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J. Immunol. 177: 7303–7311.
20. Rao, K. M. 2001. MAP kinase activation in macrophages. J. Leukoc. Biol. 69: 3–10.
Pentose phosphate cycle oxidative and nonoxidative balance: A new vulnerable target for overcoming drug resistance in cancer. *Int. J. Cancer* 119: 2733–2741.

21. Prieto, P., J. Cuenca, P. G. Través, M. Fernández-Velasco, P. Martín-Sanz, and L. Boscá. 2010. Lipoxin A4 impairment of apoptotic signaling in macrophages: implication of the PI3K/Akt and the ERK/Nrf-2 defense pathways. *Cell Death Differ.* 17: 1179–1188.

22. Martin-Sanz, P., M. Cascales, and L. Boscá. 1989. Glucagon-induced changes in fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase in cultured rat foetal hepatocytes. *Biochem. J.* 257: 795–799.

23. de Alba, P., A. Benito, P. Vizán, M. Zanuy, R. Mangues, S. Marín, and M. Cascante. 2011. Carbon metabolism and the sign of control coefficients in metabolic adaptations underlying K-ras transformation. *Biochim. Biophys. Acta* 1807: 746–754.

24. Traves, P. G., S. Hortalano, M. Zeini, T. H. Chao, T. Lam, S. T. Neuteboom, E. A. Theodorakis, M. A. Palladino, A. Castrillo, and L. Bosca. 2007. Selective activation of liver X receptors by acanthoic acid-related diterpenes. *Mol. Pharmacol.* 71: 1545–1553.

25. Lee, W. N., L. O. Byerley, E. A. Bergner, and J. Edmond. 1991. Mass isotopomer analysis: theoretical and practical considerations. *Biomed. Mass Spectrom.* 20: 451–458.

26. Schmidt, K., M. Carlsen, J. Nielsen, and J. Villadsen. 1997. Modeling isotopomer distributions in biochemical networks using isotopomer mapping matrices. *Biotechnol. Bioeng.* 55: 831–840.

27. Cascante, M., and S. Marín. 2008. Metabolomics and fluxomics approaches. *Essays Biochem.* 45: 67–81.

28. Sauer, U. 2006. Metabolic networks in motion: 13C-based flux analysis. *Mol. Syst. Biol.* 2: 62.

29. Wiechert, W., M. Moloney, S. Petersen, and A. A. de Graaf. 2001. A universal framework for 13C metabolic flux analysis. *Math. Eng.* 3: 265–283.

30. Wiechert, W., M. Moloney, N. Isermann, M. Wurzel, and A. A. de Graaf. 1999. Bidirectional reaction steps in metabolic networks: III. Explicit solution and analysis of isotopomer labeling systems. *Biotechnol. Bioeng.* 66: 69–85.

31. Wiechert, W. 2007. The thermodynamic meaning of metabolic exchange fluxes. *Biophys. J.* 93: 2255–2264.

32. Feng, G. J., H. S. Goodridge, M. M. Harnett, X. Q. Wei, A. V. Nikolaev, A. P. Higson, and F. Y. Liew. 1999. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inductible nitric oxide synthase and IL-12 in macrophages: *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* 163: 6403–6412.

33. Bustos, R., and F. Sobrino. 1992. Stimulation of glycolysis as an activation signal in rat peritoneal macrophages. Effect of glucocorticoids on this process. *Biochem. J.* 282: 299–303.

34. Newsholme, P., S. Gordon, and E. A. Newsholme. 1987. Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages. *Biochem. J.* 242: 631–636.

35. Bando, H., T. Atsumi, T. Nishio, H. Niwa, S. Mishima, C. Shimiza, N. Yoshioka, R. Bucala, and T. Kose. 2005. Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PPFKB3 family of glycolytic regulators in human cancer. *Clin. Cancer Res.* 11: 5784–5792.

36. Calvo, M. N., R. Bartrons, E. Castaño, J. C. Perales, A. Navarro-Sabaté, and A. Manzano. 2006. PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells. *FEBS Lett.* 580: 3308–3314.

37. Bensaad, K., E. C. Cheung, and K. H. Vousden. 2009. Modulation of intracellular ROS levels by TIGAR controls autophagy. *EMBO J.* 28: 3015–3026.

38. Li, H., and G. Jogl. 2009. Structural and biochemical studies of TIGAR (TP53-inducible glycolysis and apoptosis regulator). *J. Biol. Chem.* 284: 1748–1754.

39. Tomilov, A. A., V. Bicocca, R. A. Schoenfeld, M. Giorgio, E. Migliaccio, J. J. Ramsey, K. Hagopian, P. G. Pelicci, and G. A. Cortopassi. 2010. Decreased superoxide production in macrophages of long-lived p66Shc knock-out mice. *J. Biol. Chem.* 285: 1153–1165.

40. Fritz, J. M., L. D. Dwyer-Nield, and A. M. Malkinson. 2011. Stimulation of neoplastic mouse lung cell proliferation by alveolar macrophage-derived, insulin-like growth factor-1 can be blocked by inhibiting MEK and PI3K activation. *Mol. Cancer* 10: 76.

41. Barbarroja, N., R. López-Pedrera, M. D. Mayas, E. García-Fuentes, L. Garrido-Sánchez, M. Macías-Gonzalez, R. El Bekay, A. Vidal-Puig, and F. J. Tinahones. 2010. The obese healthy paradox: is inflammation the answer? *Biochem. J.* 430: 141–149.

42. Jager, J., T. Grémeaux, M. Cormont, Y. Le Marchand-Brustel, and J. P. Tanti. 2007. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 148: 241–251.

43. Kopp, A., C. Buechler, M. Bala, M. Neumeier, J. Schölmerich, and A. Schäffner. 2010. Toll-like receptor ligands cause proinflammatory and prodiabetic activation of adipocytes via phosphorylation of extracellular signal-regulated kinase and e-Jun N-terminal kinase but not interferon regulatory factor-3. *Endocrinology* 151: 1097–1108.