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Bioenergetic state regulates innate inflammatory responses through the transcriptional co-repressor CtBP

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30 **Abstract**

31 The innate inflammatory response contributes to secondary injury in brain trauma and other

32 disorders. Metabolic factors such as caloric restriction, ketogenic diet, and hyperglycemia

33 influence the inflammatory response, but how this occurs is poorly understood. Here we show

34 that glucose metabolism regulates pro-inflammatory NF-κB transcriptional activity through effects

35 on the cytosolic $NADH:NAD^+$ ratio and the NAD(H) sensitive transcriptional co-repressor, CtBP.

36 Reduced glucose availability reduces the $NADH:NAD^+$ ratio, NF-κB transcriptional activity, and pro-

37 inflammatory gene expression in macrophages and microglia. These effects are inhibited by forced

38 elevation of NADH, reduced expression of CtBP, or transfection with an NAD(H) insensitive CtBP,

39 and they are replicated by a synthetic peptide that inhibits CtBP dimerization. Changes in the

40 $NADH:NAD^+$ ratio regulate CtBP binding to the acetyltransferase p300, and regulate binding of

41 p300 and the transcription factor NF-κB to pro-inflammatory gene promoters. These findings

42 identify a mechanism by which alterations in cellular glucose metabolism can influence cellular

43 inflammatory responses.

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Microglia and macrophages exhibit a non-specific innate immune response to infection, tissue damage, and other stressors. The early phase of this response involves release of pro-inflammatory cytokines, nitric oxide, and metalloproteinases, along with alterations in cell morphology and surface protein expression [1,2]. While these responses are adaptive in the setting of infection, they can be deleterious in non-infectious injuries such as stroke and head trauma. Accordingly, factors that suppress the acute inflammatory reaction also reduce tissue loss and improve functional outcomes in animal models of non-infectious brain injury [3,4].

Inflammatory responses are influenced at the transcriptional level by factors that affect cellular bioenergetic state, such as caloric restriction, ketogenic diet, and the glycolytic inhibitor 2-deoxyglucose (2DG) [5,6]. Caloric restriction, ketogenic diet, and 2DG each produce a ketogenic state in which glucose utilization is suppressed, and these conditions also reduce brain inflammation, tissue loss, and functional impairment after brain injury [7,8,9,10,11,12]. Conversely, hypoxia and hyperglycemia promote glucose utilization, exacerbate inflammation, and worsen outcomes after brain injury [13,14].

How inflammation affects cell metabolism is well established [15], but less is known about how energy metabolism affects inflammatory responses. One potential mechanism is through the cytosolic $NADH:NAD^+$ ratio, which is thermodynamically coupled to glycolysis. The cytosolic $NADH:NAD^+$ ratio is decreased by ketogenic factors such as dietary restriction and 2DG, which decrease flux through glycolysis, and increased by conditions such as hypoxia and hyperglycemia, which increase flux through glycolysis. Cytosolic NADH and NAD^+ are physically separated from the mitochondrial metabolite pools, but can move freely across the nuclear membrane to influence transcriptional events.

Despite the fundamental roles of NAD^+ and NADH in cell metabolism, only a limited number of NADH - sensitive proteins are known to affect gene transcription [16]. Among these are the C-terminal binding proteins (CtBPs), which function as transcriptional co-repressors. Mammals express two CtBP proteins, CtBP1 and CtBP2, that exhibit overlapping actions [17,18]. CtBP forms repressor complexes with histone deacetylases, histone methyl transferases, E3 ligases, and other transcriptional regulators [19,20]. Some of these complexes include CtBP homodimers or heterodimers, while others require CtBP in its monomeric form [21,22,23,24]. CtBP in its monomeric form suppresses the activity of the acetyltransferase p300/CBP [20], which acetylates both histones

3

82 and the pro-inflammatory transcription factor, NF-κB [25]. Increased NADH levels promote the

83 formation of CtBP dimers and higher order oligomers, and thereby modulate CtBP association with

84 its binding partners [26].

85   Here we investigate whether anti-inflammatory effects of ketogenic metabolism may be

86 mediated through an NADH / CtBP signaling mechanism. We show that changes in cytosolic

87 NADH:NAD$^+$ ratio influence inflammatory responses by regulating NF-κB transcriptional activity

88 through a mechanism requiring CtBP dimerization. This process involves dissociation of p300 from

89 CtBP and acetylation of the NF-κB p65 subunit.

90

91 **Results**

92 *Microglial and macrophage activation is suppressed by 2DG*

93   The reduced glycolytic flux resulting from caloric restriction and ketogenic diet can be mimicked

94 by the glycolytic inhibitor 2-deoxyglucose (2DG) [27, 28]. To determine if 2DG can replicate the effect

95 of ketogenic diet on brain inflammatory responses, we treated rats with intraperitoneal injections

96 of lipopolysaccharide (LPS) or with LPS + 2DG. The systemic LPS injection induced a robust

97 activation of brain microglia, and this was strikingly reduced by co-administration of 2DG (Fig. 1A).

98 In organotypic brain slice cultures (Fig. 1B), 2DG likewise suppressed the effect of LPS on microglial

99 activation and on the expression of inducible nitric oxide synthase (iNOS), a hallmark of

100 inflammatory activation in microglia and macrophages [29]. Primary microglial cultures similarly

101 showed an attenuated response to LPS in the presence of 2DG (Fig. 1C), thus confirming that the

102 effects of LPS and 2DG on microglia are not dependent upon indirect, systemic effects of these

103 agents. This same pattern of responses was observed in the RAW264.7 macrophage cell line.

104 RAW264.7 cells treated with LPS showed increased iNOS mRNA expression, iNOS protein

105 expression, and nitric oxide production, all of which were attenuated by co-incubation with 2DG

106 (Fig. 1D,E). RAW264.7 cells were used because, unlike primary microglia, they can be efficiently

107 transfected and are not readily activated by transfection procedures. Glutamine was provided as

108 an alternative energy substrate in all of the *in vitro* studies, as glutamine is taken up by cells and

109 metabolized to the keto acid, α-ketoglutarate. ATP measurements confirmed no significant effect

110 of 2DG or glucose-free medium on ATP levels in the glutamine-supplemented cell cultures (Fig.

111 1F).

112   *Glycolytic inhibition reduces cytosolic NADH:NAD$^+$ ratio*

113   We assessed the free cytosolic NADH:NAD$^+$ ratio by measuring the cellular lactate:pyruvate

114   ratio [30]. This indirect approach has advantages over direct biochemical measurements in that it is

115   not influenced by protein-bound or mitochondrial NAD$^+$ and NADH [30, 31]. 2DG and glucose-free

116   medium are predicted to lower the cytosolic (i.e. non-mitochondrial) NADH:NAD$^+$ ratio by

117   decreasing glucose flux through glycolysis, while impaired respiration should increase glycolytic

118   flux and elevate the cytosolic NADH:NAD$^+$ ratio (Fig. 2A). As expected, both 2DG and glucose -free

119   medium reduced the lactate:pyruvate ratio (indicating a reduced free cytosolic NADH:NAD$^+$ ratio),

120   while the respiratory inhibitors antimycin A and cobalt chloride (CoCl$_2$) [27, 32] increased it (Fig. 2B).

121   We then evaluated these treatments on LPS-induced iNOS expression. Glucose-free medium

122   suppressed LPS-induced iNOS expression to an extent comparable to that seen with 2DG, while

123   CoCl$_2$ had the opposite effect (Fig. 2C). These findings identify a correlation between changes in

124   the inflammatory response and changes in the NADH:NAD$^+$ ratio in response to metabolic factors.

125

126   *Elevated NADH reverses the anti-inflammatory effect of 2DG*

127   We next asked whether the metabolic influences on the inflammatory responses are blocked

128   when changes in the NADH:NAD$^+$ ratio are negated. Lactate added to culture medium passes

129   readily into cultured cells [33] and drives the NADH:NAD$^+$ equilibrium toward NADH (Fig. 2A), thus

130   providing a means of countering the effect of glycolytic inhibition on the cytosolic NADH:NAD$^+$

131   ratio. The addition of sodium lactate (20 mM, pH 7.4) reversed the effects of glycolytic inhibition

132   on LPS-induced iNOS expression in the cell cultures (Fig. 3A,B). Evaluation of iNOS gene

133   transcription in cells transfected with an iNOS luciferase reporter gene confirmed an effect at the

134   level of iNOS gene transcription (Fig. 3C).

135   The lactate:pyruvate ratio cannot be used in cells treated with lactate, so for these studies we

136   instead evaluated changes in cytosolic NADH by measuring changes in intrinsic NAD(P)H

137   fluorescence. This approach does not distinguish NADH from NADPH, but cellular NADPH levels are

138   insensitive to acute changes in glycolysis or lactate [34, 35] . Signal from mitochondrial NAD(P)H was

139   avoided by sampling only from nuclei, which do not contain mitochondria (Fig. 3D). 2DG reduced

140   the cytosolic NAD(P)H signal, in agreement with the lactate:pyruvate ratio determinations; and as

141   expected 2DG had no effect on the mitochondrial NAD(P)H fluorescence (Fig. 3E). Glucose-free

142   medium had comparable effects (Fig. 3F). The effects of both 2DG and glucose-free medium on

NAD(P)H levels were reversed by sodium lactate (20 mM, pH 7.4; Fig. 3F). Although lactate did not

produce a significant increase in NAD(P)H signal when administered in the absence of 2DG (Fig.

3F), the mitochondrial inhibitors cobalt and antimycin likewise failed to increase the NAD(P)H

signal. NADH fluorescence measurements may be less sensitive to increases than to decreases in

NADH because increased NADH is disproportionately protein-unbound, and NADH that is not

protein bound has weaker intrinsic fluorescence [36].

2DG suppresses NF-κB activated pro-inflammatory genes

To evaluate the broader effects of 2DG on LPS-induced gene expression, we prepared

microarrays from RAW264.7 cells after incubation under control, LPS, or LPS+2DG conditions.

Expression levels of 994 genes were significantly affected by LPS and 781 by LPS+2DG, with 579

genes responding to both conditions (Fig. 4A). An analysis of transcription factor binding sites on

these genes showed that conserved NF-κB family binding sites (Rel and RelA/p65) were over-

represented in the LPS+2DG condition (Supplemental Table 1; Supplemental Data Sets 1 and 2), as

would be expected given the dominant role of NF-κB in pro-inflammatory transcriptional

responses. RT-PCR quantifications of specific NF-κB - driven pro-inflammatory gene transcripts,

IL1B, *IL6*, and *iNOS*, confirmed that they were upregulated by LPS, and that 2DG suppressed their

upregulation (Figs. 1D, 4B).

The anti-inflammatory effect of 2DG is mediated by CtBP

We next evaluated whether the NAD(H)-sensitive transcriptional co-repressor, CtBP, is

involved in the process by which energy metabolism influences inflammation. We generated a

stable CtBP knockdown RAW264.7 cell line using lentivirus that expressed shRNA targeting a

sequence that, in mice, is present only in *CtBP1* and *CtBP2.* The CtBP knockdown cell line displayed

a 98.9 ± 0.2 % reduction in *CtBP1* and 80.8 ± 4% reduction in *CtBP2* transcripts, and a 64 ± 10%

reduction in CtBP1/2 protein expression (Fig. 5A). Like wild-type cells, CtBP knock-down cells

responded to LPS with increased iNOS expression and NO production (though to a lesser extent);

however, unlike wild-type cells, CtBP knockdown cells showed no suppression of this response

when treated with 2DG or glucose-free medium (Fig. 5B,C). Similarly, the effect of 2DG on LPS-

induced NF-κB transcriptional activity was not observed in the CtBP knock-down cells (Fig. 5D), nor

173   was the effect of 2DG on LPS-induced *iNOS*, *Il1B*, and *Il6* gene expression (Fig. 5E; compare to Figs.

174   1D, 4B).

175   If transcriptional effects of cytoplasmic NADH : NAD$^+$ ratio are mediated by CtBP, then they

176   should be diminished in cells expressing an NAD(H) - insensitive CtBP. The G189A mutation in

177   CtBP2 disrupts its binding to NAD(H) while preserving its co-repressor activity [27]. We therefore

178   determined whether the G189A mutation would negate the effect of NADH elevations on NF-κB –

179   mediated transcriptional responses. Studies were performed in CtBP1$^{-/-}$/CtBP2$^{-/-}$ MEF cells in order

180   to eliminate effects of endogenous CtBP1/2 (Fig. 6A), and because the shRNA expressed by the

181   stable CtBP knockdown RAW264.7 cell line precludes expression of the mutant CtBP in those cells.

182   The MEF cells were transfected with either wild-type (wt) CtBP1, wt CtBP2, or mutant G189A

183   CtBP2 (Fig. 6A). Since the MEF cells responded only weakly to LPS (not shown) an NF-κB

184   transcriptional response was induced by co-transfection with the p65 subunit of NF-κB. All three of

185   the CtBP constructs suppressed NF-κB transcriptional activity, consistent with the potent co-

186   repressor activity of CtBP (Fig. 6B-D). Treatment with 2DG produced no further reduction in this

187   already suppressed NF-κB activity; however, treatment with CoCl$_2$, which elevates the NADH :

188   NAD$^+$ ratio, increased NF-κB activity in cells expressing wt CtBP2, but not in cells expressing G189A

189   mutant CtBP2 (Fig. 6E). This pattern was also observed in primary microglia: CoCl$_2$ augmented LPS-

190   induced iNOS expression in microglia overexpressing wild-type CtBP,   but not in microglia

191   overexpressing G189A CtBP2 (Fig. 6F).

192   Given that CtBP overexpression suppresses NF-κB transcriptional activity (Fig. 6C,D) we also

193   evaluated the possibility that 2DG effects on LPS-induced responses might be caused by

194   upregulated CtBP expression, independent of the CtBP responses to cytosolic NADH : NAD$^+$ ratio.

195   However, measures of CtBP mRNA levels in primary microglia showed that 2DG did not increase

196   expression of either CtBP1 or CtBP2, with or without LPS co-treatment (Suppl. Fig. 1A,B).

197

198   *Peptide inhibition of CtBP dimerization*

199   To further evaluate the mechanism by which CtBP regulates inflammatory responses, we

200   generated a synthetic peptide that blocks CtBP dimeriziation. Examination of the crystal structure

201   of a rat CtBP dimer complexed with NAD(H) [37] suggested that a peptide near the center of the

202   dimerization region, if stably folded, could bind to the CtBP monomer and interfere with dimer

203   formation. This dimerization region contains two alpha helical domains that are structurally

204   conserved in CtBP family members, and contains four residues that, when mutated, prevent

205   dimerization of drosophila CtBP [22]. A peptide spanning this region, corresponding to amino acids 114-

206   142 of the long form of mouse CtBP1 (Fig. 7A), and a control peptide lacking any known effects on

207   cell function were generated and fused to N-terminal Tat sequences (CPC Scientific (Sunnyvale,

208   CA). The CtBP peptide sequence was GRKKRRQRRRCVEETADSTLCHILNLYRRTTWLHQALREG (with

209   the Tat sequence underlined), and the control peptide sequence was

210   GRKKRRQRRRCCSFNSYELGSLCYGRKKRRQRR. To determine if the CtBP peptide could block CtBP

211   dimerization in vitro, we co-expressed CtBP1-HA and CtBP1-Flag tagged constructs in COS7 cells

212   and performed immunoprecipitation with antibody to Flag. Very little HA-tagged CtBP was

213   immunoprecipitated with anti-Flag antibody in untreated control samples (Fig. 7B), suggesting that

214   the majority of the CtBP-tagged protein is monomeric. In contrast, in vitro treatment of lysate with

215   100 mM lactate plus 10 μM NADH for 20 minutes prior to immunoprecipitation (to force an

216   increase in the $NADH:NAD^+$ ratio in the lysates) greatly increased the amount of CtBP1-HA

217   associated with CtBP-Flag. This association was disrupted by co-incubation with 50 μM of the CtBP

218   peptide (Fig. 7B).

219   We next tested whether the CtBP peptide could inhibit LPS-induced inflammatory gene

220   expression, as observed in the 2DG - induced ketogenic state. Primary microglia were treated with

221   LPS, alone or in combination with 5 μM CtBP peptide or control peptide. The CtBP peptide

222   reduced LPS-induced iNOS, IL-1b and IL-6 transcript expression, while the control peptide had no

223   effect (Fig. 7C). We then evaluated whether the CtBP peptide could block inflammatory gene

224   expression in vivo. In initial experiments, we treated mice intraperitoneally with 2DG (100 mg / kg)

225   10 minutes prior to stereotactic injection of 8 μg LPS into the left striatum. Three hours post-

226   surgery, brains were removed and microglia were isolated from the striatum for gene expression

227   studies. These studies confirmed that LPS robustly induces iNOS expression in microglia in vivo,

228   and that this effect is attenuated by 2DG (Fig. 7D). We then co-injected LPS (8 μg) along with the

229   CtBP peptide or control peptide into the striatum 3 hours prior to microglial isolation. The LPS-

230   induced increase in iNOS gene expression was reduced by the CtBP peptide, but not by the control

231   peptide. In contrast, LPS-induced expression of the M2-type anti-inflammatory gene, Socs3

232   (Suppressor of cytokine signaling 3) [38], was unaffected by CtBP peptide, demonstrating that the

233   peptide does not exert a global or nonspecific effect on transcription (Fig. 7E,F).

234

p300 binding to CtBP and pro-inflammatory gene promoters

One way that CtBP regulates gene transcription is through interactions with the histone

acetyltransferase HDAC1 [19]. We therefore performed chromatin immunoprecipitation (ChIP)

targeting the IL-6 promoter to evaluate the effects of ketogenic state on HDAC1 binding and

histone H3 acetylation. Both HDAC1 binding and H3 acetylation were increased in cells treated

with LPS, but neither was reversed by 2DG (Fig. 8A). A second way that CtBP regulates gene

transcription is by repressing the activity of p300 [20, 39], which acetylates and thereby promotes the

activity of transcription factors such as the p65 subunit of NF-κB [25]. The ChIP studies showed that

LPS induced p65 binding to the IL-6 promotor, and that this was completely reversed in the

presence of 2DG (Fig 8A). This effect of 2G was accompanied by parallel changes in p65

acetylation status, as assessed by western blots from cells treated with LPS or LPS + 2DG (Fig. 8B).

Additional ChIP studies showed that LPS induced p300 binding to the IL-6, IL-1b, and iNOS

promoter regions, and that 2DG markedly attenuated each of these effects (Fig. 8C). These results

parallel the effects of LPS and 2DG on iNOS, IL1B, and IL6 mRNA and protein expression (Figs. 1, 4).

We then performed co-immunoprecipitation of p300 with CtBP in cells transfected with either

wild-type CtBP2 or G189A CtBP2 to confirm that p300 binding to CtBP is similarly sensitive to

NADH. 2DG - induced reductions in the cytosolic NADH:NAD$^+$ ratio did not increase the binding of

p300 to either wild-type CtBP2 or G189A CtBP2, presumably because binding was already

saturated by CtBP overexpression; however, CoCl$_2$ - induced elevations in the NADH:NAD$^+$ ratio

reduced the binding of p300 to wild-type CtBP, but not G189A CtBP2 (Fig. 8D,E).

255

Discussion

CtBP has previously been shown to mediate effects of cellular metabolic state on

transcriptional events contributing to cancer and epilepsy [27, 40]. The present findings demonstrate

that CtBP similarly couples metabolic state to the innate inflammatory response. They show that

the pro-inflammatory gene expression and NF-κB transcriptional activity induced by LPS are

coupled to glucose metabolism by the cytosolic NADH:NAD$^+$ ratio, and that this coupling is

attenuated by CtBP downregulation, by forced elevation of NADH, by transfection with an NADH -

insensitive CtBP, and by a synthetic peptide that blocks NADH - induced CtBP dimerization. They

additionally show that metabolic state influences CtBP binding to the acetyltransferase p300, p300

265   binding at pro-inflammatory gene promoter sites, and acetylation state of NF-κB. The

266   observations suggest a mechanism by which the liberation of CtBP from its dimerized form by

267   lowered cytosolic NADH levels can suppress pro-inflammatory gene transcription.

268   The initial observation by Zhang et al. [26] that NADH binding promotes CtBP dimerization

269   suggested that CtBP could regulate transcription in response to metabolic changes. Subsequent

270   studies confirmed that CtBP mediates effects of 2-deoxyglucose, hypoxia, pyruvate, and other

271   metabolic influences on gene expression [27, 41]. Since NAD^+ and NADH recognize the same binding

272   site on CtBP, changes in the concentration of either nucleotide could, in principle, regulate CtBP

273   interactions with its binding partners. However, the relative changes in NADH caused by shifts in

274   the cytosolic $NADH:NAD^+$ ratio are several hundred-fold greater than the reciprocal changes in

275   NAD^+ because the cytosolic $NADH : NAD^+$ ratio is normally in the range of 1:700 [35]. The sensitivity

276   of CtBP to changes in NADH thus makes it particularly responsive to changes in energy metabolism

277   [42]. Inflammatory responses can also be modulated by NAD^+ - dependent de-acetylases of the

278   sirtuin family, notably Sirt1 [43], but unlike CtBP sirtuins are not responsive to changes in NADH

279   concentrations, and it remains uncertain whether their activity is significantly affected by the

280   relatively small changes in NAD^+ concentrations that result from changes in energy metabolism [44].

281   CtBP1 and CtBP2 have nearly identical amino acid sequences and share the same NAD(H)

282   binding site. CtBP1 lacks a putative nuclear localization signal, but may enter the nucleus as

283   heterodimer with CtBP2 or other proteins [22, 45] and may also interact in the extra-nuclear cytosol

284   with binding partners such as p300 and HDAC1. CtBP2$^{-/-}$ mice are nonviable, but CtBP1$^{-/-}$ mice are

285   viable and fertile. Mice with combinations of CtBP1 and CtBP2 mutant alleles exhibit gene dosage-

286   sensitive defects in several developmental processes [17]. Specific defects in immune function were

287   not reported in these mutants, but CtBP has elsewhere been shown to modulate the role of

288   estrogen receptors on inflammatory responses mediated by the AP-1 transcription factor,

289   independent of metabolic changes [46].

290   CtBP regulates gene transcription in multiple ways, including interactions with histone

291   deacetylases, histone methyl transferases, and E3 ligases, and the p300 acetyltransferase. We did

292   not find changes in histone acetylation or HDAC1 binding at pro-inflammatory gene promoters in

293   response to a ketogenic state, but did find decreased binding of p300 and decreased acetylation of

294   p65, which is a substrate for p300 [25]. Since p65 acetylation increases NF-κB transcriptional activity,

295   these findings identify a mechanism by which metabolic effects on CtBP can modulate gene

296   transcription. However, both monomer and dimer forms of CtBP can affect gene transcription [21, 22,

297   23, 24], and our findings do not establish that p300/CtBP binding is essential for the observed effects

298   of CtBP on pro-inflammatory gene transcription. CtBP2 could alternatively directly associate with

299   p65 and suppress its acetylation by a mechanism independent of dimer/monomer transition or

300   p300 inhibition [47]. Recent studies have also identified alternative ways that glucose metabolism

301   can influence inflammatory responses, including effects on HDAC4 protein levels, NLRP3

302   inflammasome formation, and the activation of RAGE receptors [48, 49, 50].

303   Cell cultures were used in our studies to facilitate manipulation of CtBP expression and

304   cellular cytosolic $NADH:NAD^+$ ratios. A limitation cell cultures, and cell lines in particular, is a shift

305   from respiratory ATP production toward more glycolytic ATP production (the Warburg effect [51]).

306   Moreover, the glucose concentration in standard culture media is saturating for glucose uptake

307   and metabolism. As a consequence, basal glycolytic flux is typically much higher in culture

308   preparations than in vivo. This may explain why relatively larger shifts in the cytosolic $NADH : NAD^+$

309   ratio were observed with glycolytic inhibition than with respiratory inhibitors. However, the anti-

310   inflammatory effects of 2DG in the cell culture preparations were also observed in vivo, as initially

311   reported several decades ago [6]. Importantly, these results were also mimicked, both in vitro and

312   in vivo, by the use of a novel synthetic peptide that inhibits NADH-induced CtBP dimerization. This

313   result agrees with the prior observation that native CtBP1 represses p300 activity in an NADH -

314   dependent manner, whereas dimerization-incompetent CtBP1 represses p300 activity

315   independent of NADH [20] It may be surprising that reduced CtBP expression did not itself lead to a

316   super-induction of iNOS and related genes, given co-repressor actions of CtBP. However, a

317   reduction in total CtBP does not necessarily produce a comparable reduction in nuclear

318   monomeric CtBP.

319   Taken together, our findings indicate that metabolic influences that alter the cytosolic NADH :

320   NAD^+ ratio regulate NF-κB transcriptional activity through an NADH-dependent effect on CtBP

321   dimerization. Conditions that reduce glycolytic flux, such as ketogenic diet and caloric restriction,

322   can thereby suppress NF-κB activity, while conditions that increase glycolytic flux may increase it.

323   These interactions provide a mechanism for the suppressive effects of ketogenic diet and caloric

324   restriction on brain inflammation after brain injury. By extension, these interactions may also

325   contribute to the pro-inflammatory states associated with diabetes mellitus and metabolic

326   syndrome [15, 52, 53].

327

Methods

    Reagents

    The MEF cell lines (*CtBP1$^{-/-}$/CtBP2$^{-/-}$* and *CtBP1$^{-/+}$/CtBP2$^{-/+}$*) were a kind gift from Dr. A. Roopra (University of Wisconsin, Madison). HEK293 cells and the macrophage-derived RAW264.7 cell line were obtained from the ATCC.   The p*NF-κB-RE*-firefly and pRL-CMV renilla luciferase reporter were obtained from Promega, and the control pTA-luciferase reporter was obtained from Panomics. Full-length cDNAs of mouse *CtBP1* and *CtBP2* were cloned into pIRES-hrGFP-1a (Agilent) from adult mouse brain cDNA by reverse transcriptase PCR. The CtBP2 G189A mutant was a gift from Dr. J. Blaydes (University of Southampton, UK) and was cloned into pIRES-hrGFP-1a. The *iNOS* promoter-luciferase reporter was a gift from Dr. C. Lowenstein (University of Rochester, Addgene plasmid 19296) [54], the p300 plasmid was a gift from Dr. W. Sellers (Dana-Farber Cancer Institute, Addgene plasmid 10717), the p65 plasmid was a gift from Dr. W. Greene (UCSF, Addgene plasmid 21966), and the flag-tagged CtBP2 plasmid was a gift from Dr. A. Roopra (University of Wisconsin, Madison). GIPZ-puro vector-based lentiviral vectors containing CtBP1/2 shRNA were purchased from Open Biosystem (Thermol Scientific), and purified lentivirus transduction particles were generated by the UCSF ViraCore Laboratory. All constructs were sequence verified. All other reagents were obtained from Sigma-Aldrich except where noted.

Surgical procedures and microglial isolation:

    Studies were approved by the San Francisco Veterans Affairs Medical Center animal studies committee. Male Sprague-Dawley rats, age 4-6 months (Charles River Laboratories), were given intraperitoneal injections of LPS (10 mg/kg) or saline vehicle, with or without 2-deoxyglucose (2DG; 100 mg/kg) [55]. Twenty four-hours later the rats were anesthetized and perfused with saline followed by 4% formaldehyde. Coronal 40 µm cryostat sections were prepared and immunostained[56] for CD11b (Serotec, clone OX-42, 1:100 dilution). Images were acquired using fluorescence confocal microscope and quantified as described [57].

    Stereotaxic LPS injections were performed in adult male C57BL/6J mice that were anesthetized with 2% isoflurane. The left striatum (A 1.3, ML 1.9, DV 3.5 mm from Bregma and the cortical surface) was injected with 8 µg LPS and 5 pmole CtBP peptide or control peptide in a volume of 5 µl sterile saline over 25 min (0.2 µl/min). Three hours later the mice were transcardially perfused

358   with saline to remove circulating macrophages. After perfusion, brains were quickly removed and

359   the left anterior quadrant containing the infusion site (approximately 100 mg tissue) was dissected

360   and placed in ice cold Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}. Tissue was

361   briefly minced with a sterile razor blade and cells were dissociated using Neural Tissue Dissociation

362   Kit (P) and Octo Dissociator (Miltenyi Biotec, San Diego, CA). Microglia were isolated using CD11b

363   MicroBeads (Miltenyi Biotec). Accuracy of cell separation was validated by expression profiling of

364   cellular fractions using quantitative PCR primers specific to microglia (Iba1 forward: 5'-

365   GAAGCGAATGCTGGAGAAAC, reverse: 5'-GACCAGTTGGCCTCTTGTGT), neurons (NeuN forward: 5'-

366   GGAACAGTCTATGGGCCTGA, reverse: 5'-ACAAGAGAGTGGTGGGAACG) and astrocytes (GFAP

367   forward: 5'-AGAAAGGTTGAATCGCTGGG, reverse: 5'-CGGCGATAGTCGTTAGCTTC) (Supplementary

368   Fig. 2).

369

370   *Hippocampal organotypic slice cultures*

371   Slice cultures were prepared as described [58], with minor modifications. Brains were removed

372   from 4-day old male mice C57BL/6 mice, and 350 μM sagittal sections were prepared with a

373   vibratome in ice cold dissecting media. The hippocampi were placed on Millicell inserts in 6 well

374   culture plates, and maintained for 2 weeks at 37˚C in 5% CO_2/ 95% air incubator. Experiments

375   were initiated by adding 10 μg / ml LPS ± 1 mM 2DG. After 24 hours the slices were fixed with 4%

376   formaldehyde. Slices were immunostained using rabbit polyclonal antibody to Iba1 (Wako #019-

377   19741; 1:200 dilution) and rabbit polyclonal antibody to iNOS (Millipore, # 06-573; 1:250 dilution).

378

379   *Cell cultures*

380   Mouse primary microglia cells were isolated and cultured as described [56] and used after 10

381   days in vitro. Cell lines were seeded at a density of 1×10^4 cells / well in 24-well plates one day

382   prior to the experiments. For protein expression and luciferase assays, MEF cells, RAW264.7 cells

383   and HEK293 cells were seeded in 12-well or 24-well plates overnight, and transfected with

384   plasmids using Lipofectamine 2000 (Invitrogen) when they reached 80% confluence. A stable

385   CtBP1/2 knockdown cell line was generated from RAW264.7 cells by incubating the cells with

386   CtBP1/2 shRNA lentiviral stocks for 3 hours. Puromycin was added after 48 hours, and infected

387   cells were selected over 10 passages. Stable clones were pooled for propagation and experiments.

13

388   Studies were initiated by washing cells into serum-free Dulbecco's minimal essential medium

389   containing 6 mM glutamine and 2 mM glucose (or no glucose, where indicated).

390   Lipopolysaccharide (LPS), lactate, and metabolic inhibitors were added to this medium from

391   concentrated stocks that were pre-adjusted to pH 7.4, and the cultures were replaced in a 37° C,

392   5% CO_2 incubator. The final LPS concentration was 10 ng / ml in the cell cultures. All experiments

393   were repeated at least 3 times, using triplicate wells in each experiment.

394

395   *ATP measurements*

396   Cells were washed with ice cold phosphate-buffered saline and extracted with ice cold 0.5%

397   trichloroacetic acid. The cell lysate pH was adjusted to 7.8 with 1M Tris base. ATP concentration

398   was determined by a luciferase-linked method (Promega Enlighten ATP kit) using ATP standards.

399   Values were normalized to protein concentrations as determined by the Bradford assay (Bio-Rad)

400   in sister culture wells.

401

402   *Lactate and pyruvate assays*

403   RAW264.7 cells were rinsed with ice-cold phosphate-buffered saline, lysed in 0.2 N NaOH, and

404   aliquots were taken for protein assay. Lactate and pyruvate were measured in pH-neutralized

405   lysates by enzyme-linked assays as described [12] using standards treated identically.

406

407   *NADH imaging*

408   Real-time changes in cellular (cytoplasmic) NADH content were estimated by measuring

409   endogenous NADH fluorescence with 360 nm excitation and > 410 nm emission [59]. Fluorescence

410   images were acquired at 1 minute intervals after 5 minutes of baseline recording. Regions of

411   interest were selected from the nucleus to eliminate signal from mitochondria, and cytochalasin D

412   (1 µM) was included to prevent cell movement. Images acquired from each cell were used to

413   calculate change in fluorescence intensity / average of pre-treatment fluorescence intensity

414   ($\Delta F/F_o$). In a subset of experiments cells were pre-incubated with 200 nM Mitotracker Red-FM

415   (Molecular Probes) to verify that the NADH fluorescence measurements were not contaminated

416   by mitochondrial NADH fluorescence. Mitochondrial and NADH signals were imaged by

417   interleaving NADH fluorescence excitation and Mitotraker (excitation 550 nm; emission > 610 nm).

418

Nitric oxide measurements

Production of nitric oxide was assessed by analyzing nitrite concentrations in the culture
medium collected after 24 hours incubation under the designated conditions. Aliquots of culture
medium or nitrite standards prepared in culture medium were mixed with equal volumes of Griess
reagent and light absorbance was measured at 540 nm [56]. Values were normalized to the protein
content of each culture well.

Luciferase reporter gene assays

Cells were transfected with 300 ng of *5xNF-κB* or *iNOS* firefly luciferase constructs using
Lipofectamine 2000 (Life Technologies), and co-transfected with 100 ng of GL3-Renilla luciferase
construct (Promega) as an internal control of transfection efficiency. In some experiments cells
were also transfected with a CtBP constructs (200 ng) or CtBP empty control vector. Renilla
luciferase and firefly luciferase activity were measured in cell lysates using a dual luciferase assay
kit (Promega) on a Modulus Microplate Reader (Turner Biosystems). Reporter luciferase activity
was normalized to Renilla luciferase activity in each assay.

Microarray and differential expression analysis

Cells were harvested after 24 hours incubation under the designated conditions. RNA was
extracted and arrayed in triplicates using Mouse OneArray chip (Phalanx Biotech). Data analysis
was performed using Bioconductor packages in R (www.bioconductor.org). Raw reads from
microarrays were first normalized across the samples using the 'vsn' package from Bioconductor
[60]. The normalized expression levels of the samples (with technical and experimental duplicates)
were then fitted with a mixed linear model using the 'limma' package from Bioconductor [61].
Differential expression between pairs of treatment factors were also calculated using the 'limma'
package, and the resulting p-values were adjusted for multiple tests using the Benjamin-Hochberg
procedure. A Venn diagram was also generated to visualize the overlap of differentially expressed
genes between pairs of factors among three factors, using R codes adapted from the 'limma'
package. Shared transcription factor binding sites were identified by blast differentially expressed
genes in the oPOSSUM server (http://opossum.cisreg.ca/oPOSSUM3/) with a filter setting of Z-
score above 15.

449

Quantitative real-time PCR analysis

RNA was extracted from freshly harvested cells (Qiagen), treated with DNAse I (Promega) and first-strand cDNA was synthesized from 2 µg total RNA with Oligo (dT) primers (Life Technologies). Real-time PCR with SYBR green detection (Applied Biosystems) was performed as described previously [62] using an ABI 7900HT FAST Real-Time PCR System. Dilution series and standard curves of beta-actin were amplified on each plate for all experiments. Transcript levels of all genes from each sample were normalized to its ß-Actin mRNA level using the $2\text{-}\Delta\Delta^{CT}$ method [62].

Co-immunoprecipitation and western blots

Cells were lysed with M-PER reagent (Pierce) supplemented with benzonase nuclease (Novagen) and protease inhibitors (Roche). Soluble lysate was centrifuged for 10 minutes at 4°C and the supernatant was incubated with anti-Flag M2 agarose (Sigma) overnight. The M2 agarose was washed 5 times with 0.25% Triton X-100 / phosphate-buffered saline and 3 times with 0.5% Triton X-100 / phosphate-buffered saline. Bound protein was eluted using 1X Sample Loading Buffer (Invitrogen) and heated to 100°C for 5 minutes. Lysate and eluate were resolved on 10% SDS-PAGE gels, and transferred to PVDF membranes (Millipore) [62]. The blots were probed with mouse polyclonal anti iNOS (Upstate, #06-573; 1:1000), rabbit polyclonal anti ß-actin (Sigma-Aldrich, #A2066; 1:1000), and mouse monoclonal anti-CtBP (AbNova, # H1487-MO1, 1:1000).

Chromatin immunoprecipitation

Cells were treated with 1% formaldehyde for 10 minutes at 37°C and rinsed twice with ice-cold phosphate-buffered saline supplemented with phenylmethanesulfonyl fluoride and protease inhibitors (Roche). Cells were then lysed, sonicated on ice, and centrifuged. Aliquots of the supernatants were heated to reverse crosslinks and recover genomic DNA for an input control. A ChIP assay kit (Millipore) was used with mouse antibody to CtBP (AbNova, # H1487-MO1, 1:250 dilution) and rabbit antibodies to p300, HDAC1, and p65 (Santa Cruz Biotechnology; sc-585, sc-7872, sc-372) or acetyl H3 (Cell Signaling, #8173), each at 1:250 dilutions, and with mouse and rabbit IgG controls (Cell Signaling). Precipitated material was eluted by two 15 minute incubations at room temperature with 250 µl of 1% SDS / 0.1 M NaHCO$_3$. Chromatin was crosslink-reversed and submitted to RNase and proteinase K digestion. DNA was extracted by phenol-chloroform and

480   the DNA from both the ChIP and the input controls were analyzed by quantitative real-time PCR

481   analysis. Primers targeting the iNOS, Il-1b, and Il6 promoter regions were as published [63, 64].

482

483   *Peptide design and synthesis*

484   The crystal structure of a rat CtBP dimer complexed with NAD(H) (PDB 1HKU)[37] using Accelrys

485   DS Viewer 1.7 suggested that a peptide near the center of the dimerization region, if stably folded,

486   might interfere with dimer formation. This dimerization region contains two alpha helical domains

487   that are structurally conserved in CtBP family members and also contains residues that, when

488   mutated, prevent dimerization of drosophila CtBP [22]. A corresponding CtBP peptide along with a

489   control peptide lacking any known cell function were generated and fused to an N-terminal Tat

490   sequence by CPC Scientific (Sunnyvale, CA). The CtBP peptide sequence was

491   <u>GRKKRRQRRRC</u>VEETADSTLCHILNLYRRTTWLHQALREG (with the Tat sequence underlined), and the

492   control peptide sequence was <u>GRKKRRQRRRC</u>CSFNSYELGSLCYGRKKRRQRR.

493

494   *CtBP dimerization assay*

495   CtBP1-HA and CtBP1-Flag expression constructs were generated by PCR amplification of full

496   length CtBP1 coding sequence from mouse brain-derived cDNA, followed by cloning in-frame into

497   pSelect-CHA-zeo (Invivogen, San Diego, CA) and pCMV-(DYKDDDDK)-C (Clontech, Mountain View,

498   CA) vectors, respectively. Clone identity and integrity were verified by sequence analysis. COS-7

499   cells were grown to 75% confluence in 6-well plates in Dulbecco's minimal essential medium

500   containing fetal bovine serum and 230 µM sodium pyruvate. 5 µg of each plasmid per plate was

501   transfected using Lipofectamine2000 (Life Technologies, Inc., Grand Island, NY). 24 hours post

502   transfection, cells were washed once in 1 x PBS and lysed in NP-40 buffer (20 mM Tris HCl pH 8.0,

503   137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2mM EDTA) containing Complete Mini protease

504   inhibitor cocktail (Roche, Indianapolis, IN). Lysates were cleared of cellular debris by brief

505   centrifugation and concentration determined by Pierce BCA Protein Assay (Thermo Scientific,

506   Rockford, IL). Forty µg protein from each sample lysate was incubated at 37° C for 20 minutes

507   either with or without the addition of 50 µM CtBP peptide. In some studies incubation solution

508   also contained 10 mM sodium lactate and 10 µM NADH to promote CtBP dimerization. Co-

509   immunoprecipitation was performed by overnight incubation of samples with 2 µg mouse

510   monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich, # F3165), 3hr incubation with 40 µL

511 prewashed protein A/G-Sepharose beads (Biovision Inc., Milpitas, CA) and serially washed with NP-

512 40 buffer per manufacturer's instructions. Bound protein was eluted from Sepharose beads by

513 addition of NuPAGE LDS Sample buffer (Life Technologies, Inc.) and analyzed by standard SDS-

514 PAGE gel electrophoresis using polyclonal rabbit anti-HA antibody (Thermo Scientific, # PA1-985,

515 1:500 dilution).

516

517 *Statistical analyses.*

518   In all cell culture or slice culture studies the 'n' values denote the number of independent

519 experiments, each using neurons prepared from different mice. Each independent experiment

520 contained triplicate culture wells. Data other than the microarray results are expressed as means ±

521 SEM and assessed using one-way ANOVA followed by either the Tukey–Kramer test where

522 multiple groups are compared against one another, or Dunnett's test where multiple groups are

523 compared against a common control group. All data analyses were performed while blinded to the

524 treatment conditions.

525

526   *Data availability*

527   The microarray data have been deposited in the FigShare database at

528 https://doi.org/10.6084/m9.figshare.c.3828235.v1. The authors declare that all other relevant

529 data supporting the findings of the study are available in this article and its Supplementary

530 Information files, or from the corresponding author upon request.

References

1. Brown GC, Neher JJ. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. *Mol Neurobiol* **41**, 242-247 (2010).

2. Chen Y, Won SJ, Xu Y, Swanson RA. Targeting Microglial Activation in Stroke Therapy: Pharmacological Tools and Gender Effects. *Curr Med Chem* **21**, 2146-2155 (2014).

3. An C*, et al.* Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. *Prog Neurobiol* **115**, 6-24 (2014).

4. Loane DJ, Byrnes KR. Role of microglia in neurotrauma. *Neurotherapeutics* **7**, 366-377 (2010).

5. Longo VD, Mattson MP. Fasting: molecular mechanisms and clinical applications. *Cell Metab* **19**, 181-192 (2014).

6. Goth A. Inhibition of anaphylactoid edema in the rat by 2-deoxyglucose. *Am J Physiol* **197**, 1056-1058 (1959).

7. Gasior M, Rogawski MA, Hartman AL. Neuroprotective and disease-modifying effects of the ketogenic diet. *Behav Pharmacol* **17**, 431-439 (2006).

8. Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. *J Neurosci Res* **57**, 830-839 (1999).

9. Loncarevic-Vasiljkovic N*, et al.* Caloric restriction suppresses microglial activation and prevents neuroapoptosis following cortical injury in rats. *PLoS One* **7**, e37215 (2012).

10. Tu YF, Lu PJ, Huang CC, Ho CJ, Chou YP. Moderate dietary restriction reduces p53-mediated neurovascular damage and microglia activation after hypoxic ischemia in neonatal brain. *Stroke* **43**, 491-498 (2012).

11. Appelberg KS, Hovda DA, Prins ML. The effects of a ketogenic diet on behavioral outcome after controlled cortical impact injury in the juvenile and adult rat. *J Neurotrauma* **26**, 497-506 (2009).

12. Wang Q*, et al.* Pyruvate protects against experimental stroke via an anti-inflammatory mechanism. *Neurobiol Dis* **36**, 223-231 (2009).

559   13.   Kaur C, Rathnasamy G, Ling EA. Roles of activated microglia in hypoxia induced

560   neuroinflammation in the developing brain and the retina. *J Neuroimmune Pharmacol* **8**,

561   66-78 (2012).

562   14.   Robbins NM, Swanson RA. Opposing effects of glucose on stroke and reperfusion injury:

563   acidosis, oxidative stress, and energy metabolism. *Stroke* **45**, 1881-1886 (2014).

564   15.   Hotamisligil GS. Inflammation and metabolic disorders. *Nature* **444**, 860-867 (2006).

565   16.   Ghosh S, George S, Roy U, Ramachandran D, Kolthur-Seetharam U. NAD: a master regulator

566   of transcription. *Biochim Biophys Acta* **1799**, 681-693 (2010).

567   17.   Hildebrand JD, Soriano P. Overlapping and unique roles for C-terminal binding protein 1

568   (CtBP1) and CtBP2 during mouse development. *Mol Cell Biol* **22**, 5296-5307 (2002).

569   18.   Chinnadurai G. Transcriptional regulation by C-terminal binding proteins. *Int J Biochem Cell*

570   *Biol* **39**, 1593-1607 (2007).

571   19.   Kuppuswamy M*, et al.* Role of the PLDLS-binding cleft region of CtBP1 in recruitment of

572   core and auxiliary components of the corepressor complex. *Mol Cell Biol* **28**, 269-281

573   (2008).

574   20.   Kim JH, Cho EJ, Kim ST, Youn HD. CtBP represses p300-mediated transcriptional activation

575   by direct association with its bromodomain. *Nat Struct Mol Biol* **12**, 423-428 (2005).

576   21.   Birts CN*, et al.* A cyclic peptide inhibitor of C-terminal binding protein dimerization links

577   metabolism with mitotic fidelity in breast cancer cells. *Chem Sci* **4**, 3046-3057 (2013).

578   22.   Bhambhani C, Chang JL, Akey DL, Cadigan KM. The oligomeric state of CtBP determines its

579   role as a transcriptional co-activator and co-repressor of Wingless targets. *EMBO J* **30**,

580   2031-2043 (2011).

581   23.   Verger A*, et al.* Mechanisms directing the nuclear localization of the CtBP family proteins.

582   *Mol Cell Biol* **26**, 4882-4894 (2006).

583   24.   Quinlan KG*, et al.* Role of the C-terminal binding protein PXDLS motif binding cleft in

584   protein interactions and transcriptional repression. *Mol Cell Biol* **26**, 8202-8213 (2006).

585   25.   Chen LF, Mu Y, Greene WC. Acetylation of RelA at discrete sites regulates distinct nuclear

586   functions of NF-kappaB. *EMBO J* **21**, 6539-6548 (2002).

587   26.   Zhang Q, Piston DW, Goodman RH. Regulation of corepressor function by nuclear NADH.

588   *Science* **295**, 1895-1897 (2002).

589　27.　Garriga-Canut M*, et al.* 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-
590　　　 dependent metabolic regulation of chromatin structure. *Nat Neurosci* **9**, 1382-1387 (2006).

591　28.　Yao J, Chen S, Mao Z, Cadenas E, Brinton RD. 2-Deoxy-D-glucose treatment induces
592　　　 ketogenesis, sustains mitochondrial function, and reduces pathology in female mouse
593　　　 model of Alzheimer's disease. *PLoS One* **6**, e21788 (2011).

594　29.　Forstermann U, Kleinert H. Nitric oxide synthase: expression and expressional control of
595　　　 the three isoforms. *Naunyn Schmiedebergs Arch Pharmacol* **352**, 351-364 (1995).

596　30.　Williamson DH, Lund P, Krebs HA. The redox state of free nicotinamide-adenine
597　　　 dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem J* **103**, 514-527 (1967).

598　31.　Alano CC, Tran A, Tao R, Ying W, Karliner JS, Swanson RA. Differences among cell types in
599　　　 NAD(+) compartmentalization: A comparison of neurons, astrocytes, and cardiac myocytes.
600　　　 *J Neurosci Res* **85**, 3378-3385 (2007).

601　32.　Karovic O*, et al.* Toxic effects of cobalt in primary cultures of mouse astrocytes. Similarities
602　　　 with hypoxia and role of HIF-1alpha. *Biochem Pharmacol* **73**, 694-708 (2007).

603　33.　Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across
604　　　 mammalian plasma membranes. *Am J Physiol* **264**, C761-782 (1993).

605　34.　Reiss PD, Zuurendonk PF, Veech RL. Measurement of tissue purine, pyrimidine, and other
606　　　 nucleotides by radial compression high-performance liquid chromatography. *Anal Biochem*
607　　　 **140**, 162-171 (1984).

608　35.　Ying W. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation
609　　　 and biological consequences. *Antioxid Redox Signal* **10**, 179-206 (2008).

610　36.　Palero JA, Bader AN, de Bruijn HS, der Ploeg van den Heuvel A, Sterenborg HJ, Gerritsen
611　　　 HC. In vivo monitoring of protein-bound and free NADH during ischemia by nonlinear
612　　　 spectral imaging microscopy. *Biomed Opt Express* **2**, 1030-1039 (2011).

613　37.　Nardini M*, et al.* CtBP/BARS: a dual-function protein involved in transcription co-repression
614　　　 and Golgi membrane fission. *EMBO J* **22**, 3122-3130 (2003).

615　38.　Qin H*, et al.* Signal transducer and activator of transcription-3/suppressor of cytokine
616　　　 signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation. *Proc Natl*
617　　　 *Acad Sci U S A* **109**, 5004-5009 (2012).

618　39.　Meloni AR, Lai CH, Yao TP, Nevins JR. A mechanism of COOH-terminal binding protein-
619　　　 mediated repression. *Mol Cancer Res* **3**, 575-583 (2005).

620 40. Di LJ, Fernandez AG, De Siervi A, Longo DL, Gardner K. Transcriptional regulation of BRCA1

621   expression by a metabolic switch. *Nat Struct Mol Biol* **17**, 1406-1413 (2011).

622 41. Zhang Q, Wang SY, Nottke AC, Rocheleau JV, Piston DW, Goodman RH. Redox sensor CtBP

623   mediates hypoxia-induced tumor cell migration. *Proc Natl Acad Sci U S A* **103**, 9029-9033

624   (2006).

625 42. Fjeld CC, Birdsong WT, Goodman RH. Differential binding of NAD+ and NADH allows the

626   transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic

627   sensor. *Proc Natl Acad Sci U S A* **100**, 9202-9207 (2003).

628 43. McGettrick AF, O'Neill LA. How metabolism generates signals during innate immunity and

629   inflammation. *J Biol Chem* **288**, 22893-22898 (2013).

630 44. Feldman JL, Dittenhafer-Reed KE, Denu JM. Sirtuin catalysis and regulation. *J Biol Chem*

631   **287**, 42419-42427 (2012).

632 45. Bergman LM, Morris L, Darley M, Mirnezami AH, Gunatilake SC, Blaydes JP. Role of the

633   unique N-terminal domain of CtBP2 in determining the subcellular localisation of CtBP

634   family proteins. *BMC Cell Biol* **7**, 35 (2006).

635 46. Saijo K, Collier JG, Li AC, Katzenellenbogen JA, Glass CK. An ADIOL-ERbeta-CtBP

636   transrepression pathway negatively regulates microglia-mediated inflammation. *Cell* **145**,

637   584-595 (2011).

638 47. Xu X*, et al.* EVI1 acts as an inducible negative-feedback regulator of NF-kappaB by inhibiting

639   p65 acetylation. *J Immunol* **188**, 6371-6380 (2012).

640 48. Moon JS*, et al.* mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3

641   Inflammasome Activation. *Cell Rep* **12**, 102-115 (2015).

642 49. Wang B*, et al.* Glycolysis-dependent histone deacetylase 4 degradation regulates

643   inflammatory cytokine production. *Mol Biol Cell* **25**, 3300-3307 (2015).

644 50. Manigrasso MB, Juranek J, Ramasamy R, Schmidt AM. Unlocking the biology of RAGE in

645   diabetic microvascular complications. *Trends Endocrinol Metab* **25**, 15-22 (2014).

646 51. Warburg O. On respiratory impairment in cancer cells. *Science* **124**, 269-270 (1956).

647 52. Calle MC, Fernandez ML. Inflammation and type 2 diabetes. *Diabetes Metab* **38**, 183-191

648   (2012).

649 53. Horvath P, Oliver SR, Ganesan G, Zaldivar FP, Jr., Radom-Aizik S, Galassetti PR. Fasting

650   glucose level modulates cell surface expression of CD11b and CD66b in granulocytes and

651   monocytes of patients with type 2 diabetes. *J Investig Med* **61**, 972-977 (2013).

652 54. Lowenstein CJ*, et al.* Macrophage nitric oxide synthase gene: two upstream regions

653   mediate induction by interferon gamma and lipopolysaccharide. *Proc Natl Acad Sci U S A*

654   **90**, 9730-9734 (1993).

655 55. Suh SW*, et al.* Glucose and NADPH oxidase drive neuronal superoxide formation in stroke.

656   *Ann Neurol* **64**, 654-663 (2008).

657 56. Kauppinen TM, Higashi Y, Suh SW, Escartin C, Nagasawa K, Swanson RA. Zinc triggers

658   microglial activation. *J Neurosci* **28**, 5827-5835 (2008).

659 57. d'Avila JC*, et al.* Microglial activation induced by brain trauma is suppressed by post-injury

660   treatment with a PARP inhibitor. *Journal of neuroinflammation* **9**, 31 (2012).

661 58. del Rio JA, Soriano E. Regenerating cortical connections in a dish: the entorhino-

662   hippocampal organotypic slice co-culture as tool for pharmacological screening of

663   molecules promoting axon regeneration. *Nat Protoc* **5**, 217-226 (2010).

664 59. Brennan AM, Connor JA, Shuttleworth CW. NAD(P)H fluorescence transients after synaptic

665   activity in brain slices: predominant role of mitochondrial function. *J Cereb Blood Flow*

666   *Metab* **26**, 1389-1406 (2006).

667 60. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization

668   applied to microarray data calibration and to the quantification of differential expression.

669   *Bioinformatics* **18 Suppl 1**, S96-104 (2002).

670 61. Smyth GK. Linear models and empirical bayes methods for assessing differential expression

671   in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3 (2004).

672 62. Shen Y*, et al.* Mutations in PNKD causing paroxysmal dyskinesia alters protein cleavage and

673   stability. *Hum Mol Genet* **20**, 2322-2332 (2011).

674 63. Saijo K*, et al.* A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic

675   neurons from inflammation-induced death. *Cell* **137**, 47-59 (2009).

676 64. Zschiedrich I*, et al.* Coactivator function of RIP140 for NFkappaB/RelA-dependent cytokine

677   gene expression. *Blood* **112**, 264-276 (2008).

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680

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Author Contributions

Y.S. and R.A.S. conceived the project. Y.S., D.K., A.M.B-M., and R.A.S. designed the experiments; Y.S., D.K., J-E.K., L.H.L. and Y.C. evaluated inflammatory responses, CtBP expression, and performed CtBP immunoprecipitation studies in the cell cultures; A.M.B-M. performed the fluorescence NADH analyses and ATP measurements; Y. S. and Y.H. provided data analysis of the microarray and promoter enrichment studies; S.J.W. performed the in vivo studies; S.M.M. designed the CtBP peptide; D.K. established and performed the dimerization assay with the CtBP peptide; and Y.S., D.K., and R.A.S wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

700

FIGURE LEGENDS

Figure 1. 2-deoxyglucose suppresses LPS-induced microglial activation.

703 (A) Immunostaining for CD11b identifies activated microglia in rat hippocampus. CD11b expression

704 was increased 24 hours after intraperitoneal injection with LPS (10 mg / kg). The increase was

705 attenuated by co-injection with 2-deoxyglucose (2DG; 100mg / kg). Scale bar = 100 µm.   ** p <

706 0.01, n = 6.   (B) Immunostaining for Iba1 and iNOS identify activated microglia in mouse

707 hippocampal slice cultures after 24 hours incubation with LPS (10 µg /ml) or LPS +2DG (1 mM).

708 Scale bar = 100 µm; n $\geq$ 3, * p < 0.05. Culture medium contained 6 mM glutamine and 5 mM

709 glucose. (C) Effects of 1mM 2DG on LPS (10 ng / ml) - induced iNOS transcript and protein

710 expression in primary microglial cultures. n = 5; * p < 0.05, ** p < 0.01. Full length immunoblots

711 are shown in Supplementary Fig. 3. (D,E). Effects of 1 mM 2DG on LPS -induced iNOS protein

712 expression, mRNA expression, and nitric oxide production in cultured RAW267.4 cells. n $\geq$ 3; *p <

713 0.05, **p < 0.01.   (F) Relative ATP levels measured after 24 hours incubation in control medium, 1

714 mM 2-deoxyglucose, 200 µM $CoCl_2$, 20 mM lactate, glucose free medium, or in 2 µM

715 trifluorocarbonylcyanide phenylhydrazone (FCCP) as a positive control. n = 4; ** p < 0.01 v.

716 control. Error bars show s.e.m.

717

718 **Figure 2.   Relationships between glucose metabolism and cytosolic NADH:NAD$^+$ ratio**.

719 (A) Factors that reduce glucose flux through glycolysis, such as reduced glucose availability or

720 glycolytic inhibitors, reduce NADH levels and thereby reduce NADH:NAD$^+$ ratio, whereas factors

721 that inhibit oxidative metabolism, such as hypoxia and mitochondrial inhibitors, have the opposite

722 effect. Glutamine provides ketone bodies (α-ketoglutarate) to fuel mitochondrial ATP production

723 in the absence of glycolysis. Lactate dehydrogenase (LDH) maintains the lactate:pyruvate ratio in

724 equilibrium with the cytosolic NADH:NAD$^+$ ratio. (B) The lactate:pyruvate ratio provides an index

725 of the cytosolic NADH:NAD$^+$ ratio in cells treated with glycolytic and mitochondrial inhibitors. 2DG,

726 1 mM 2-deoxyglucose; 0 Glu, glucose-free medium; $CoCl_2$, 200 µM cobalt chloride; antimycin, 1

727 µM antimycin A.   n = 4; *p < 0.05 v. control. (C) LPS-induced iNOS expression was suppressed in

728 RAW267.4 cells treated with 1 mM 2DG or glucose-free medium, and increased in cells treated

729 with the mitochondrial inhibitor cobalt chloride ($CoCl_2$, 200 µM). n = 4; * p < 0.05 v. control. Error

730 bars show s.e.m. Full length immunoblots are shown in Supplementary Fig. 3.

731

732 **Figure 3. Lactate reverses the effects of glycolytic inhibition on both cytosolic NADH levels and**

733 **LPS-induced iNOS expression.**

734 (A,B) The effects of glucose-free medium and 2DG on LPS-induced iNOS expression are reversed by

735 20 mM lactate. $n \geq 3$, *$p < 0.05$. Full length immunoblots are shown in Supplementary Fig. 3. (C)

736 The effects of glucose-free medium and 2DG on LPS-induced *iNOS* transcription are reversed by 20

737 mM lactate, as measured by relative light units (RLU) emitted by a cells transfected with a

738 luciferase-coupled *iNOS* reporter gene. $n = 3$; * $p < 0.05$. (D) Effects of lactate on cytosolic

739 NAD(P)H levels as measured by intrinsic fluorescence (blue). Mitochondria are labeled with

740 Mitotracker (red). Lower row images are enlarged views of areas defined by rectangle in upper

741 row. Boxes in lower row identify a mitochondria-rich peri-nuclear region and a mitochondria-free

742 nuclear region in one cell. (E) Example of real-time NAD(P)H fluorescence changes recorded from

743 these two regions during incubation with 1 mM 2-deoxyglucose (added at arrow). (F) Quantified

744 results showing relative cytosolic NAD(P)H fluorescence changes induced by incubation with 1 mM

745 2-deoxyglucose ± 20 mM lactate, glucose-free medium ± 20 mM lactate, 20 mM lactate, 200 µM

746 cobalt chloride, or 1 µM antimycin A. $n = 5$; * $p < 0.01$, #$p < 0.01$ v. control. Error bars show s.e.m.

747

748 **Figure 4. LPS and 2-deoxyglucose influence NF-κB – mediated gene transcription.**

749 (A) Microarray analysis identified 994 genes differentially regulated by LPS (red) and 781 genes by

750 (2DG+LPS) (blue), relative to control conditions. 579 of LPS-response genes were affected by co-

751 incubation with 2DG. (B) RT-PCR measures of NF-κB –driven pro-inflammatory cytokines

752 confirmed that LPS-induced induction was attenuated by 2DG. *IL-1b,* interleukin-1beta; *IL-6,*

753 interleukin-6. $n \geq 3$; * $p < 0.05$, ** $p < 0.01$. Error bars show s.e.m.

754

755 **Figure 5. Knockdown of CtBP eliminates the effects of 2DG and glucose-free medium.**

756 (A) Representative western blot showing reduced expression of CtBP1/2 protein in RAW264.7 cells

757 transfected with shRNA targeting CtBP1 and CtBP2 (CtBP KD). Full length immunoblots are shown

758 in Supplementary Fig. 3. (B,C) shRNA knockdown of CtBP1/2 negates the effect of both 2DG and

759 glucose-free medium on LPS-induced iNOS expression and nitric oxide production. Results for wild-

760 type (WT) cells were normalized to control (no LPS) WT cells, and results for CtBP knockdown cells

761 (CtBP KD) were normalized to control CtBP KD cells. $n = 4$; *$p < 0.05$; ns, not significant. Error bars

762 show S.E.M.   (D) Knockdown of CtBP1/2 negates the effects of 2DG on LPS-induced NF-κB

763 reporter gene activation. $n \geq 3$; *$p < 0.05$; ns, not significant. Error bars show s.e.m. (E) 2DG did

764 not suppress LPS-induced transcription of *iNOS*, *Il-1b*, or *IL-6* in the CtBP KD cells. n = 3. (Compare

765 to Figs. 1D, 4B).

766

767 **Figure 6. The NAD(H) binding site on CtBP is required for its effect on inflammatory responses.**

768 (A) Transfection with CtBP1, CtBP2, and G1892 CtBP2 produced comparable expression levels in

769 CtBP1$^{-/-}$/CtBP2$^{-/-}$ MEF cells. Full length immunoblots are shown in Supplementary Fig. 3. (B-D) MEF

770 cells were transfected with WT CtBP1, CtBP2 and G189A CtBP2, and additionally transfected with

771 p65 to induce NF-κB activation. All 3 CtBP constructs suppress *iNOS* and *NF-κB* reporter gene

772 transcriptional activity, and have no effect on a scrambled-sequence driven luciferase reporter

773 gene. n =3; *p < 0.05 v. empty vector. (E) The mitochondrial inhibitor CoCl$_2$ increased *NF-κB*

774 reporter gene activity in cells expressing WT CtBP2 but not in cells expressing G189A CtBP2.

775 Results are normalized to the increase produced by CoCl$_2$ in the cells transfected with empty

776 vector alone. n = 4; *p < 0.05. (F) Immunostaining in primary microglia shows LPS-induced iNOS

777 expression is potentiated by CoCl$_2$ in cells transfected with WT CtBP2, but not G189A CtBP. Larger

778 nuclei in the images belong to the astrocyte feeder layer. Results are normalized to the increase

779 produced by 200 μM CoCl$_2$ in the empty vector - transfected cells. n = 4; **p < 0.01. Error bars

780 show s.e.m.

781

782 **Figure 7. Direct inhibition of CtBP dimerization blocks LPS-induced pro-inflammatory gene**

783 **expression.**

784 (A) Schematic of CtBP1 protein showing functional domains and alignment with the CtBP peptide

785 used to block CtBP dimerization. PLDLS indicates substrate binding domain. The CtBP blocking

786 peptide includes an N-terminal TAT sequence for cellular internalization. (B) Immunoprecipitation

787 assay showing ability of CtBP peptide (50 μM) to block dimerization of tagged CtBP proteins

788 incubated with 10 mM lactate + 10 μM NADH. Full length immunoblots are shown in

789 Supplementary Fig. 3. Immunoprecipitation was performed with anti-Flag antibody, and CtBP1-

790 Flag/CtBP1-HA heterodimers were detected by western blots using anti-HA antibody. Lysate

791 control lane = 5% of input used in immunoprecipitated samples. n = 4. **p < 0.01. (C) CtBP peptide

792 (5 μM) blocks LPS-induced mRNA expression of pro-inflammatory genes (iNOS, IL-1b and IL-6) in

793 cultured primary microglia. Ctrl PEP = control peptide. n ≥ 3; **p < 0.01. (D) 2DG blocks LPS-

794 induced iNOS gene expression in microglia isolated from mice after LPS (8 μg) injection into

795   striatum. n = 3-5 per group. **p < 0.01. (E,F) CtBP peptide blocks LPS-induced iNOS expression but

796   not Socs3 expression in brain microglia. n = 4 per group. **p < 0.01. Error bars show s.e.m.

797

798   **Figure 8. CtBP effects on p300 and NF-κB acetylation**

799   (A) Chromatin immunoprecipitation (ChIP) with antibody to HDAC1, acetyl-H3, and p65 was

800   performed in RAW264.7 cells to evaluate binding to the IL-6 promoter regions. LPS increased all

801   three signals, but only the effect of p65 binding was reversed by 2DG (conditions as in Fig. 1C); n =

802   3, *p < 0.05. (B) Western blots show LPS-induced p65 acetylation is suppressed by 2DG

803   (conditions as in Fig. 1C); n = 3, *p < 0.05. Full length immunoblots are shown in Supplementary

804   Fig. 3. (C) ChIP was performed to evaluate p300 binding to NF-κB p65 binding sites on promoter

805   regions of pro-inflammatory cytokines. p300 binding was increased by LPS, and this effect was

806   attenuated by 2DG (conditions as in Fig. 1C); n = 3, *p < 0.05. (D) HEK293 cells were transfected

807   with FLAG-tagged wild-type CtBP2 or G189A CtBP2. Immunoprecipitation using antibody to FLAG

808   recovered p300 protein, while antibody to IgG, used as a negative control, did not.

809   Immunoprecipitates from transfected cells treated with 2DG or $CoCl_2$ for 30 minutes showed

810   reduced p300 binding to wt CtBP2, but not G189A CtBP2, in cells treated with $CoCl_2$. (E) Quantified

811   results of the immunoprecipitation studies. Results were normalized to p300 in the lysate input. n

812   = 3; *, p < 0.05. Error bars show s.e.m.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

A

CtBP1/2

β-Actin

WT | CtBP KD

B

<u>Wt cells</u>

Ctrl LPS | LPS+ 2DG | LPS+ 0 Glucose

iNOS

β-actin

iNOS expression

Ctrl | LPS | LPS+ 2DG | LPS+ 0 Glucose

<u>CtBP KD cells</u>

Ctrl LPS | LPS+ 2DG | LPS+ 0 Glucose

iNOS

β-actin

N.S.

iNOS expression

Ctrl | LPS | LPS+ 2DG | LPS+ 0 Glucose

C

Nitrite accumulation

■ LPS
■ LPS+2DG

*
ns

WT | CtBP KD

Nitrite accumulation

■ LPS
■ LPS+0 Glucose

*
ns

WT | CtBP KD

D

NF-kB-Luciferase

RLU

■ LPS
■ LPS+2DG

*
ns

WT | CtBP KD

E

<u>CtBP KD cells</u>

Fold change

iNOS

Control | LPS | LPS+ 2DG

Fold change

IL-1b

Control | LPS | LPS+ 2DG

Fold change

IL-6

Control | LPS | LPS+ 2DG

Figure 6

A

CtBP1⁻/⁻, CtBP2⁻/⁻ MEF cells

Empty Vector   CtBP1   CtBP2   CtBP2 G189A

CtBP

β-Actin

B *iNOS*-Luciferase

C *NF-kB*-Luciferase

D Scramble-Luciferase

E *NF-kB*-Luciferase

F Primary microglia

CtBP-Flag/iNOS/DAPI

Figure 7

A

CtBP1

dimerization

PLDLS

1 27 121 181-204 327 352 440

NAD(H) binding

TAT VEETADSTLCHILNLYRRTTWLHQALREG CtBP peptide

125 153

B

IP: Flag

Relative HA binding

lysate / IP Ctrl / IP dimerized / IP dimerized + CtBP PEP

** **

IP: Flag

WB: HA

lysate / IP Ctrl / IP dimerized / IP dimerized + CtBP PEP

C

iNOS mRNA

Fold Change

Ctrl / LPS / LPS+ CtBP PEP / LPS+ Ctrl PEP

** **

IL-1b mRNA

Fold Change

Ctrl / LPS / LPS+ CtBP PEP / LPS+ Ctrl PEP

** **

IL-6 mRNA

Fold Change

Ctrl / LPS / LPS+ CtBP PEP / LPS+ Ctrl PEP

** **

D

iNOS mRNA

Fold Change

Ctrl / LPS / LPS+ 2DG

**

E

iNOS mRNA

Fold Change

Sham / LPS+ Ctrl PEP / LPS+ CtBP PEP

**

F

Socs3 mRNA

Fold Change

Sham / LPS+ Ctrl PEP / LPS+ CtBP PEP

* *

Figure 8

A ChIP IL-6

Ab: HDAC1 — Fold Change (Ctrl, LPS, LPS+2DG) ** **

Ab: acetyl-H3 — Fold Change (Ctrl, LPS, LPS+2DG) ** *

Ab: p65 — Fold Change (Ctrl, LPS, LPS+2DG) ** **

B

iNOS, Acetyl-p65, p65, β-actin (Ctrl, LPS, LPS+2DG)

Acetyl-p65 — Relative level (Ctrl, LPS, LPS+2DG) *

C

ChIP iNOS — Ab: p300 — Fold change (Ctrl, LPS, LPS+2DG) *

ChIP IL-1b — Ab: p300 — Fold change (Ctrl, LPS, LPS+2DG) *

ChIP IL-6 — Ab: p300 — Fold change (Ctrl, LPS, LPS+2DG) *

D

IP: Flag, WB: p300 (IgG, CtBP)

WB: Lysate p300

IP: FLAG (Control, 2DG, CoCl₂) (Empty Vector, CtBP2, CtBP2 G189A)

E

Control — Relative p300 binding (Empty Vector, CtBP2, CtBP2 G189A) N.S.

2DG — Relative p300 binding (Empty Vector, CtBP2, CtBP2 G189A) N.S.

CoCl₂ — Relative p300 binding (Empty Vector, CtBP2, CtBP2 G189A) *