Nonsteroidal Anti-Inflammatory Drug Flufenamic Acid Is a Potent Activator of AMP-Activated Protein Kinase

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ABSTRACT

Flufenamic acid (FFA) is a nonsteroidal anti-inflammatory drug (NSAID). It has anti-inflammatory and antipyretic properties. In addition, it modulates multiple channel activities. The mechanisms underlying the pharmacological actions of FFA are presently unclear. Given that AMP-activated protein kinase (AMPK) has both anti-inflammatory and channel-regulating functions, we examined whether FFA induces AMPK activation. 1) Exposure of several different types of cells to FFA resulted in an elevation of AMPKα phosphorylation at Thr172. This effect of FFA was reproduced by functionally and structurally similar mefenamic acid, tolfenamic acid, niflumic acid, and meclofenamic acid. 2) FFA-induced activation of AMPK was largely abrogated by the treatment of cells with 1,2-bis(2-amino-5-methylphenyl)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (an intracellular Ca2⁺ chelator) or depletion of extracellular Ca2⁺, whereas it was mimicked by stimulation of cells with the Ca2⁺ ionophore 5-(methylamino)-2-((2R,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-[(1H-pyrrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-1,3-benzoxazole-4-carboxylic acid acetate; SC-514, 4-amino-257-(methylamino)-2-((2R,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-[(1H-pyrrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-1,3-benzoxazole-4-carboxylic acid); 3) FFA triggered a rise in intracellular Ca2⁺, which was abolished by cyclosporine, a blocker of mitochondrial permeability transition pore. Cyclosporine also abolished FFA-induced activation of AMPK. 4) Inhibition of Ca2⁺/calmodulin-dependent kinase kinase (CaMKKβ) with 7-oxo-7H-benzimidazo[2,1-a]benz[de]soquinoline-3-carboxylic acid acetate (STO-609) or down-regulation of CaMKKβ with short interfering RNA largely abrogated FFA-induced activation of AMPK. 5) FFA significantly suppressed nuclear factor-κB activity and inducible nitric-oxide synthase expression triggered by interleukin-1β and tumor necrosis factor α. This suppression was also largely abrogated by STO-609. Taken together, we conclude that FFA induces AMPK activation through the Ca2⁺/CaMKKβ pathway. Activation of AMPK is a presently unrecognized important mechanism underlying the pharmacological effects of FFA.

Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase composed of a catalytic α subunit and regulatory β and γ subunits. It serves as a sensor of the energy state of the cells and is a key regulator of metabolic homeostasis (Towler and Hardie, 2007). AMPK is activated in response to increased AMP/ATP ratio, as well as by phosphorylation of Thr172 on its catalytic subunit and by direct binding of the upstream kinases LKB1 (liver kinase B1) and Ca2⁺/calmodulin-dependent kinase kinase (CaMKK) (Shaw et al., 2004; Hawley et al., 2005). Once activated, AMPK switches off ATP-consuming pathways, thereby promoting energy conservation and cell survival. Flufenamic acid (FFA) is a potent activator of AMPK in various types of cells.

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**Fig. 1.** Effects of fenamates on AMPK activation. A, effects of structurally similar fenamates on the activation of AMPK. NRK-52E cells were exposed to 50 μM FFA, MFA, TFA, NFA, or MCFA for 5 min. Cellular protein was extracted and subjected to Western blot analysis for the phosphorylated levels of AMPKa, AMPKβ, and ACC. The equal loading of protein in each lane was verified by probing the blots with an anti-β-actin antibody. Results are representative of three separate experiments. B, densitometric analysis of the phosphorylated AMPKa shown in A. Results are expressed as relative unit (mean ± S.E.; n = 3; *, P < 0.01 versus untreated control (Con)). C to F, time-course and concentration-dependent effects of FFA on AMPK. NRK-52E cells were exposed to 50 μM FFA for the indicated time intervals (C and D) or different concentrations of FFA for 5 min (E and F). Cellular protein was extracted and subjected to Western blot analysis. D and F, densitometric analysis of the phosphorylated AMPKa shown in C and E, respectively (mean ± S.E.; n = 3; *, P < 0.01 versus untreated control).

**Fig. 2.** Effects of FFA on activation of AMPK in several different cell lines and tissues. A to F, effects of FFA on activation of AMPK in several different cell lines. Pig kidney proximal tubular epithelial cells (LLC-PK1; A), the mouse hepatoma cell line Hepa 1c1c-7 (B), mouse preadipocyte 3T3-L1 cells (C), human HeLa cells (D), and human prostate cancer epithelial LNCaP (E) and PC-3 cells (F) were exposed to the indicated concentrations of FFA for 5 min. The phosphorylation level of AMPKa at Ser172 was determined by Western blot. β-Actin levels shown at the bottom of the blots indicate the same amount of loading of the protein. G and H, effects of FFA on AMPK activation in vivo. Mice were intraperitoneally injected with the indicated concentrations of FFA for 30 min. Proteins from liver (G) and kidney (H) tissues were extracted and subjected to Western blot analysis for the phosphorylated AMPKa. β-Actin levels shown at the bottom of the blots indicate the same amount of loading of the protein. Results are representatives of two to three separate experiments.
pathways and switches on ATP-producing pathways. In addition to its regulatory functions on cellular metabolic pathways, AMPK has anti-inflammatory effects (Pilon et al., 2004; Cheng et al., 2007; Jeong et al., 2009; Aoki et al., 2010; Cai et al., 2010; Shin et al., 2010). In addition, it promotes angiogenesis, protects cells from apoptosis (Shaw et al., 2004), and modulates a variety of channel activities (Carattino et al., 2005; Mace et al., 2008; Klein et al., 2009; Kongsuphol et al., 2009; Kréneisz et al., 2009). Flufenamic acid (FFA) is one of the nonsteroidal anti-inflammatory drugs (NSAIDs) used for the alleviation of inflammation and pain in the clinic (Flower et al., 1972). In addition, FFA regulates multiple channel activities. FFA, on the one hand, inhibits gap junction channels (Harks et al., 2001), Ca\(^{2+}\)-activated chloride channels (White and Aylwin, 1990), cystic fibrosis transmembrane conductance regulator chloride channels (McCarty et al., 1993), voltage-gated sodium channels (Yau et al., 2010), transient receptor potential (TRP) channels (Hill et al., 2010), and nonselective cation channels (Poronnik et al., 1992). On the other hand, it activates potassium channels (Ottolia and Toro, 1994), TRPC6 channels (Foster et al., 2009), and TRPA1 channels (Hu et al., 2010). The channel-regulating property of FFA has been extensively exploited for both experimental and therapeutic purposes in a variety of pharmacologic and pathophysiological models.

At present, little is known about the molecular mechanisms underlying the actions of FFA. Several studies have shown that FFA is able to elevate intracellular Ca\(^{2+}\) (McDougall et al., 1988; Poronnik et al., 1992; Jordani et al., 2000; Gardam et al., 2008; Tu et al., 2009). Furthermore, a possible link between FFA-induced elevation of intracellular Ca\(^{2+}\) and the alterations of channel activities has been proposed (Poronnik et al., 1992; Gardam et al., 2008). However, the downstream molecular events implicated in the actions of FFA are still poorly understood. Several considerations prompted us to speculate a possible involvement of AMPK. First, AMPK can be activated by Ca\(^{2+}\) through the CaMKK\(^{β}\) pathway (Stahmann et al., 2006). Second, similar to FFA, AMPK has both anti-inflammatory and channel-regulatory activity (Pilon et al., 2004; Carattino et al., 2005; Cheng et al., 2007; Mace et al., 2008; Jeong et al., 2009; Klein et al., 2009; Kongsuphol et al., 2009; Kréneisz et al., 2009; Aoki et al., 2010; Cai et al., 2010; Shin et al., 2010). For example, both FFA and AMPK have been reported to suppress the inflammatory mediator-induced expression of inducible nitric-oxide synthase (iNOS) (Paik et al., 2000; Aoki et al., 2010) and inhibit sodium channel (Carattino et al., 2005; Yau et al., 2010). Third, FFA is reported to inhibit glucose production and promote glucose glycolysis in a model of isolated perfused liver (Lopez et al., 1998). These metabolic changes could also be achieved through the activation of AMPK (Towler and Hardie, 2007). Therefore, the purpose of this study was to determine whether FFA could induce AMPK activation.

Here, we present evidence showing that FFA potently activates AMPK through the Ca\(^{2+}\)-CaMKK\(^{β}\) pathway. Activation of AMPK is a presently unrecognized important mechanism underlying the pharmacological actions of FFA.

### Materials and Methods

**AMPK**

**FFA + Cyclosporin**

**FFA**

**Materials.** IL-1β and TNFα were purchased from R&D Systems (Minneapolis, MN). 4-Amino-[2',3'-bithiophene]-5-carboxamide (SC-514), aspirin, and anti-iNOS antibody were obtained from Cayman Chemical (Ann Arbor, MI). Antiphospho-AMPKα (Thr172), antiphospho-AMPKγ1 (Ser108), and antiphospho-acetyl-coA carboxylase (ACC; Ser79) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Fetal bovine serum (FBS), trypsin/EDTA, anti-biots, anti-β-actin antibodies, and all other chemicals were purchased from Sigma (Tokyo, Japan).

**Cells.** Normal rat kidney proximal epithelial cells (NRK-52E), the porcine kidney epithelial cell line LLC-PK1, the mouse hepatoma cell line Hepa 1c1c-7, human epithelial carcinoma cell lines (HeLa, PC-3, and LNCaP), and the mouse preadipocyte cell line 3T3-L1 were purchased from the American Type Culture Collection (Manassas, VA). For maintenance, these cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 5 to 10% FBS. For experiments, they were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 1% FBS.

**Fig. 3.** Induction of intracellular Ca\(^{2+}\) by FFA and its prevention by cyclosporine. NRK-52E cells were exposed to 30 μM FFA in the presence of absence of 5 μM cyclosporine for the indicated seconds. The results are presented as dynamic traces of Ca\(^{2+}\) over time (A), representing average levels of intracellular Ca\(^{2+}\) among 15 to 20 cells in a single study, or intracellular Ca\(^{2+}\) level at basal and peak values after the addition of FFA (B). Ca\(^{2+}\) concentration is expressed as the ratio of emitted fluorescence at 340 and 380 nm (F340/F380). *P < 0.01 versus FFA alone (mean ± S.E.; n = 15–20).
Animals. Adult female C57BL/6J mice, weighting 25 to 30 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Animal experimental plans and procedures were approved by the Animal Experimental Committee of Yamanashi University.

Western Blot Analysis. Total cellular protein was extracted by suspending the prewashed cells in SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol) with freshly added proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Lysates were incubated on ice for 30 min with intermittent mixing and then centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was recovered and protein concentration was determined using the Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Western blot was performed using the enhanced chemiluminescence system (Yao et al., 2005). In brief, extracted cellular proteins were separated by 10% or 4 to 20% gradient SDS-polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in phosphate-buffered saline, the membranes were incubated with antiphospho-AMPKα and -AMPKβ, antiphospho-ACC, or anti-iNOS antibody. After washing, the membranes were probed with horseradish peroxidase-conjugated anti-rabbit IgG, and the bands were visualized using the enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, U.K.).

Fig. 4. Modulation of intracellular Ca2+ on FFA-induced activation of AMPK. A, induction of AMPKα phosphorylation by Ca2+ inophores A23187 and ionomycin. NRK-52E cells were exposed to 5 μM A23187 and 10 μM ionomycin for 5 min. B to D, FFA-induced activation of AMPK in the absence of extracellular Ca2+ or the presence of the Ca2+ chelator BAPTA-AM. NRK-52E cells were either cultured in Ca2+-free medium (B and C) or normal Ca2+ medium with 100 μM BAPTA-AM (D) for 1 h before exposure to FFA for an additional 5 min. C, densitometric analysis of the blot shown in B. Data are expressed as the percentage of FFA-stimulated levels of p-AMPK (mean ± S.E.; n = 3). *, P < 0.05 versus FFA alone. E and F, effects of the inhibition of mitochondria permeability transition pore with cyclosporine on FFA-induced activation of AMPK. E, NRK-52E cells were pretreated with 5 μM cyclosporine for 30 min, and then exposed to 50 μM FFA for an additional 5 min. Cellular proteins were extracted and subjected to Western analysis for the phosphorylated AMPKα. β-Actin shown at the bottom of the blot indicates the same amount of loading of protein. F, densitometric analysis of the results of E. Data are expressed as the percentage of FFA-stimulated levels of p-AMPK (mean ± S.E.; n = 3). *, P < 0.05 versus FFA alone.
Buckinghamshire, UK). The chemiluminescent signal was captured with a Fujifilm luminescent image LAS-1000 analyzer (Fujifilm, Tokyo, Japan) and quantified with densitometric software. To confirm equal loading of proteins, the membranes were probed for β-actin protein.

Measurement of Ca\textsuperscript{2+}. Cultured NRK-52E cells were loaded with fura-2 by incubation with 5 μM fura-2 acetoxymethyl ester in Hanks’ balanced salt solution containing 2.0 mM CaCl\textsubscript{2} and 1 mM MgCl\textsubscript{2} at room temperature. Ca\textsuperscript{2+} was determined by the ratio method as reported previously (Yao et al., 2003).

Transient Transfection of Cells with siRNA. NRK-52E cells were transiently transfected with siRNA specifically targeting CaMKKβ or a negative control siRNA (AllStars Negative Control siRNA; QIAGEN, Tokyo, Japan) at a final concentration of 20 nM with Fugene transfection reagent for 48 h. After that, cells were left untreated or exposed to 50 μM FFA for 5 min. Cellular proteins were extracted and analyzed for phosphorylated AMPKα.

Establishment of Stable Transfectant. NRK/NF\textsubscript{kappaB}-SEAP reporter cells were established by stably transfection of NRK-52E cells with pNF\textsubscript{kappaB}-SEAP (BD Biosciences, San Jose, CA) as described previously (Yao et al., 2003). pNF\textsubscript{kappaB}-SEAP encodes SEAP under the control of NF\textsubscript{kappaB}.

SEAP Assay. Activity of SEAP in culture media was evaluated by a chemiluminescent method using the Great EscAPe SEAP detection kit (BD Biosciences) as described previously (Yao et al., 2005; Hayakawa et al., 2006a). Activity was determined by the ratio method as reported previously (Yao et al., 2003).

Measurement of Nitrite Levels. NO production was assayed by detecting nitrite accumulation in the culture medium using Griess reagent (Green et al., 1982). In brief, 100 μl of a solution containing 1% sulfanilamide and 0.1% naphthylethylenediamine in 2 M HCl was added to 100 μl of conditioned medium. Samples were incubated at room temperature for 10 min, and then the absorbance was measured with a microtiter plate reader at 550 nm. Nitrite levels were expressed in picomoles of NO\textsubscript{2} per microgram of total cellular protein.

Statistical Analysis. Values are expressed as mean ± S.E. Comparison of two populations was made by Student’s t test. For multiple comparisons with a single control, one-way analysis of variance followed by Dunnett’s test was used. Both analyses were carried out using SigmaStat statistical software (Systat Software, Inc., San Jose, CA). P < 0.05 was considered to be a statistically significant difference.

Fig. 5. Involvement of CaMKKβ in FFA-induced activation of AMPK. A and B, abrogation of FFA-induced activation of AMPKα by kinase inhibitors. NRK-52E cells were pretreated with 50 nM calphostin (A) or 5 μM STO-609 (B) for 15 min, and then exposed to 50 μM FFA for an additional 5 min. C, densitometric analysis of blot shown in B. Data are expressed as the percentage of FFA-stimulated levels of p-AMPKα (mean ± S.E.; n = 3). * P < 0.05 versus FFA alone. D, inhibition of FFA-induced activation of AMPKα by specific siRNA against CaMKKβ. NRK-52E cells were transfected with CaMKKβ siRNA or control siRNA for 48 h. After that, cells were exposed to 50 μM FFA for 5 min. Cellular proteins were extracted and subjected to Western analysis for phosphorylated AMPKα and CaMKKβ. Equal loading of protein per lane was verified by probing the blot with an anti-β-actin antibody. Results are representatives of two to three separate experiments.
Fig. 6. Suppressive effects of FFA on cytokine-induced iNOS expression and NFkB activation. A, suppression of cytokine-induced iNOS expression by FFA and AICAR. NRK-52E cells were stimulated with 2 ng/ml IL-1β and 20 ng/ml TNFα in the presence or absence of 50 μM FFA or 500 μM AICAR for 24 h. Cellular proteins were extracted and subjected to Western analysis for iNOS. Equal loading of protein per lane was verified by probing the blot with an anti-β-actin antibody. B, densitometric analysis of iNOS expression shown in A. Results are expressed as the percentage of the cytokine-stimulated level of iNOS (mean ± S.E.; n = 3). *, P < 0.05 versus the cytokine-stimulated cells. C, attenuation of FFA-induced suppression
Results

FFA Induces AMPK Activation. FFA is one of the N-ary-anthranilic acid derivatives, belonging to the fenamate group of NSAIDs. Other members of the fenamate group of NASIDs are mefenamic acid (MFA), tolfenamic acid (TFA), niflumic acid (NFA), and meclofenamic acid (MCPA). All of these chemicals have similar structures and functions (Winder et al., 1963; Poronnik et al., 1992). To test whether fenamates activate AMPK, we examined the influence of these chemicals on phosphorylation levels of AMPK at Thr172 in NRK-52E cells. Previous studies had established that phosphorylation of this site correlates with AMPK activity (Towler and Hardie, 2007). As shown in Fig. 1A and B, incubation of NRK cells with fenamates resulted in increased levels of phosphorylated AMPKα and AMPKβ, which was associated with parallel elevation of phosphorylated ACC, one of the AMPK substrates (Towler and Hardie, 2007). Densitometric analysis of the blots in Fig. 1A revealed that all the chemicals significantly activated AMPK. Among them, the effects of FFA and TFA were more pronounced (Fig. 1B). Considering the widespread use of FFA in a variety of in vivo and in vitro experimental systems, we chose FFA for further analysis.

Time-course analysis of the effect using FFA revealed that the activation of AMPK was rapid, which was detectable as early as 2 min after FFA addition, peaked at approximately 15 min, and was retained at a relatively high level for at least 12 h (Fig. 1, C and D). Concentration-effect analysis revealed that the activation of AMPK was concentration-dependent. The clear activation could be observed at the concentrations of FFA as low as 10 μM (Fig. 1, E and F). These results thus indicate that N-ary-anthranilic acid derivative is a novel class of AMPK activator.

Activation of AMPK by FFA Is Not Cell Type- and Species-Specific. To determine whether the effect of FFA is cell type- and species-specific, we evaluated AMPK activation in several different types of cells. As shown in Fig. 2, FFA caused concentration-dependent activation of AMPK in pig kidney proximal tubular epithelial cells (LLC-PK1; Fig. 2A), the mouse hepatoma cell line Hepa 1c1c-7 (Fig. 2B), the mouse preadipocyte cell line 3T3-L1 (Fig. 2C), human HeLa cells (Fig. 2D), and human prostate cancer epithelial cells (LNCaP and PC-3; Fig. 2, E and F, respectively).

FFA also induced AMPK activation in vivo. Intraperitoneal injection of FFA into mice caused an elevation of AMPK phosphorylation in both liver (Fig. 2G) and kidney (Fig. 2H) tissues. These results indicate that the effect of FFA is not cell type- and species-specific and can be detected both in vitro and in vivo.

FFA-Induced Activation of AMPK Depends on Intracellular Ca²⁺. Several studies have demonstrated that FFA is able to elevate intracellular Ca²⁺ through the induction of Ca²⁺ releases from mitochondria (McDougall et al., 1988; Poronnik et al., 1992; Jordani et al., 2000; Gardam et al., 2008; Tu et al., 2009). We, therefore, evaluated the role of Ca²⁺ in FFA-induced activation of AMPK. First, we confirmed the calcium-elevating effect of FFA in NRK-52E cells. Consistent with previous reports (McDougall et al., 1988; Poronnik et al., 1992; Jordani et al., 2000; Gardam et al., 2008; Tu et al., 2009), FFA elevated intracellular Ca²⁺ in NRK-52E cells (Fig. 3). This effect was completely blocked by cyclosporine, an inhibitor of the mitochondria permeability transition pore (MPTP) (Broekeemeier and Pfeiffer, 1995).

We then examined the role of the elevated intracellular Ca²⁺ in AMPK activation. As shown in Fig. 4A, induction of intracellular Ca²⁺ with the Ca²⁺ ionophores 5-(methylamino)-2-[(2R,3R, 6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)methyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-1,3-benzoazol-4-carboxylic acid (A23187) and ionomycin increased phosphorylation levels of AMPKα. In contrast, inhibition of intracellular Ca²⁺ by culture of cells in a calcium-free medium or addition of the Ca²⁺ chelator BAPTA-AM largely abrogated AMPK activation (Fig. 4, B-D). Consistent with the causative role of MPTP opening in FFA-induced elevation in intracellular Ca²⁺, cyclosporine also significantly blocked AMPK activation (Fig. 4, E and F). Thus the elevated Ca²⁺ is required for FFA-induced activation of AMPK.

CaMKβ Underlies FFA-Induced Activation of AMPK. Increased intracellular Ca²⁺ activates various kinases, including a well documented AMPK, CaMKβ (Hawley et al., 2005). To assess the role of kinases, especially CaMKβ, we examined the influence of the PKC inhibitor calphostin and a specific CaMKβ inhibitor 7-oxo-1H-benzimidazo[2,1-a]benz[de]isoquinoline-3-carboxylic acid acetate (STO-609) on the activation of AMPK. As shown in Fig. 5, A to C, both agents effectively suppressed AMPK phosphorylation. Furthermore, down-regulation of CaMKβ with the specific siRNA also abolished the effect of FFA (Fig. 5D).

AMPK Contributes to FFA-Induced Suppression of NFκB Activity and iNOS Expression. In addition to its crucial role in the control of metabolic processes, AMPK suppresses inflammatory responses (Cheng et al., 2007; Peairs et al., 2009). Therefore, we tested the possible implication of AMPK on the anti-inflammatory effect of FFA. For this purpose, we examined proinflammatory cytokines IL-1β and TNFα-induced expression of iNOS. As shown in Fig. 6, A and B, FFA markedly inhibited the cytokine-induced expression of iNOS in NRK-52E cells. This effect was similarly produced by a well known AMPK activator, 5-aminooimidazole-4-carboxamide-1β,8-diborufosanoside (AICAR), suggesting a possible involvement of AMPK. Because activation of AMPK by FFA in NRK-52E cells was mediated by CaMKβ, we, therefore, determined the role of AMPK by inhibition of iNOS by inhibition of the CaMKβ. NRK-52E cells were pretreated with 5 μM STO-609 for 15 min and then exposed to 2 ng/ml IL-1β plus 20 ng/ml TNFα in the presence of absence of FFA for 24 h. D, densitometric analysis of iNOS expression shown in C. Results are expressed as the percentage of the cytokine-stimulated level of iNOS (mean ± S.E.; n = 3), *, P < 0.05. NS, not significantly different (P > 0.05). E, abrogation of FFA-induced suppression of NO formation by inhibition of the AMPK CaMKβ. NRK-52E cells were treated as in D. The conditioned media were harvested for measurement of nitrite levels. Data are expressed as mean ± S.E. (n = 4), *, P < 0.05, NS, not significantly different (P > 0.05). F, role of NFκB in cytokine-induced iNOS expression. NRK cells were treated with the NFκB inhibitor SC-514 (100 μM) for 30 min and then exposed to 2 ng/ml IL-1β and 20 ng/ml TNFα for 24 h. Cellular proteins were extracted and subjected to Western blot analysis using an anti-iNOS antibody. Expression of β-actin was used as loading control. G, abrogation of FFA-induced suppression of NFκB activity by inhibition of the CaMKβ. NRK/NFκB-SEAP reporter cells were exposed to 2 ng/ml IL-1β and 20 ng/ml TNFα in the presence or absence of 50 μM FFA and/or 5 μM STO-609 for 24 h. The conditioned media were harvested for measurement of SEAP activity. Data are expressed as mean ± S.E. (n = 4), *, P < 0.05.
CaMKKβ. As shown in Fig. 6, C to E, the CaMKKβ inhibitor STO-609 significantly abrogated the suppressive effect of FFA on iNOS expression and NO formation.

One recent study indicated that AMPK suppresses iNOS expression through the inhibition of NFκB (Aoki et al., 2010). We, therefore, examined the possible effect of FFA on NFκB. First, we confirmed that the cytokine-induced expression of iNOS was controlled by NFκB. Inhibition of NFκB with SC-514 completely abrogated cytokine-elicited iNOS expression (Fig. 6F). To determine the influence of FFA on NFκB, we transfected NRK-52E cells with pNFκB-SEAP and monitored SEAP activity in the conditioned media. As shown in Fig. 6G, FFA significantly inhibited cytokine-induced NFκB activation, which was also significantly blocked by STO-609. These observations indicate that FFA suppresses iNOS expression through CaMKKβ-dependent inhibition of NFκB.

**Discussion**

In this study, we provide the first evidence showing that FFA and other members of the fenamate class of NSAIDs are potent activators of AMPK. The mechanisms involved are schematically illustrated in Fig. 7. Given that FFA has been widely used as an anti-inflammatory and channel-regulating agent in clinical and basic research, our findings may have significant implications for understanding the pharmacological actions of FFA.

FFA induced AMPK activation through the Ca^{2+}-CaMKKβ pathway. This is shown by the fact that activation of AMPK by FFA was Ca^{2+}-dependent and abolished by inhibition or down-regulation of CaMKKβ. Apart from CaMKKβ, AMPK is also phosphorylated by LKB1 (Shaw et al., 2004). However, it is less likely that LKB1 played a major role in this study, because FFA similarly triggered AMPK activation in LKB1-deficient Hela cells (Fig. 2D) (Shaw et al., 2004).

CaMKK is a Ca^{2+}-dependent kinase. It is activated subsequent to the elevation of intracellular Ca^{2+} (Hawley et al., 2005; Stahmann et al., 2006). Indeed, FFA elicited a rise in intracellular Ca^{2+} in NRK-52E cells. Consistent with previous reports (McDougall et al., 1988; Poronnik et al., 1992; Jordani et al., 2000; Tu et al., 2009), treatment of cells with FFA caused inhibition in ATP production (Lopez et al., 1998; Tu et al., 2009). Given that the increased AMP/ATP ratio can allosterically activate AMPK (Towler and Hardie, 2007), an involvement of this mechanism in this study is also likely. It is noteworthy that LKB1 played a major role in this study, because FFA has been recognized to be causative of the reduced biogenesis in mitochondria (Cárdenas et al., 2010). Therefore, it can be said that FFA-induced activation of AMPK resides in its ability to alter Ca^{2+} concentrations inside and outside of mitochondria.

One previous report demonstrated that PKC mediated the ischemic precondition-induced activation of AMPK (Nishino et al., 2004). It is noteworthy that activation of AMPK by FFA was also abolished by calphostin C, a widely used inhibitor of protein kinase C. In this context, a possible involvement of PKC in this study cannot be excluded. However, calphostin C has also been described to be able to block L-type Ca^{2+} channels (Nishino et al., 2004). It is unclear whether the effect of calphostin C was caused by its inhibition on PKC or intracellular Ca^{2+}. More detailed analysis on this aspect may be needed in the future.

It is noteworthy that in this study we observed that the different types of cells varied in their response to FFA-induced activation of AMPK. For example, FFA induced a detectable activation of AMPK in NRK-52E cells at a concentration as low as 5 to 10 μM. In contrast, the similar extent of activation in LLC-PK1 cells required more than 50 μM FFA. The reasons for the discrepancy are unclear. It could be related to the different capacity of the cells in uptaking, processing, and metabolizing FFA. It could also be caused by the difference in the abundance of CaMKKβ among the different types of cells. It should be mentioned that FFA at the concentrations used in this study did not exhibit significant cytotoxic effect, as revealed by lactate dehydrogenase releasing and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (data not shown).

FFA has multifaceted functions. It is conceivable that the effect of FFA on AMPK activation could be a secondary event resulting from its effects on other target molecules. However, the mechanisms by which FFA alters mitochondria Ca^{2+} fluxes are still unclear and needed to be clarified in the future. FFA has also been reported to uncouple mitochondria (McDougall et al., 1988; Poronnik et al., 1992; Jordani et al., 2000; Gardam et al., 2008; Tu et al., 2009). Treatment of cells with FFA caused inhibition in ATP production (Lopez et al., 1998; Tu et al., 2009). Given that the increased AMP/ATP ratio can allosterically activate AMPK (Towler and Hardie, 2007), an involvement of this mechanism in this study is also likely. It is noteworthy that the loss of mitochondria Ca^{2+} has been recognized to be causative of the reduced biogenesis in mitochondria (Cárdenas et al., 2010). Therefore, it can be said that FFA-induced activation of AMPK resides in its ability to alter Ca^{2+} concentrations inside and outside of mitochondria.

One previous report demonstrated that PKC mediated the ischemic precondition-induced activation of AMPK (Nishino et al., 2004). It is noteworthy that activation of AMPK by FFA was also abolished by calphostin C, a widely used inhibitor of protein kinase C. In this context, a possible involvement of PKC in this study cannot be excluded. However, calphostin C has also been described to be able to block L-type Ca^{2+} channels (Nishino et al., 2004). It is unclear whether the effect of calphostin C was caused by its inhibition on PKC or intracellular Ca^{2+}. More detailed analysis on this aspect may be needed in the future.

It is noteworthy that in this study we observed that the different types of cells varied in their response to FFA-induced activation of AMPK. For example, FFA induced a detectable activation of AMPK in NRK-52E cells at a concentration as low as 5 to 10 μM. In contrast, the similar extent of activation in LLC-PK1 cells required more than 50 μM FFA. The reasons for the discrepancy are unclear. It could be related to the different capacity of the cells in uptaking, processing, and metabolizing FFA. It could also be caused by the difference in the abundance of CaMKKβ among the different types of cells. It should be mentioned that FFA at the concentrations used in this study did not exhibit significant cytotoxic effect, as revealed by lactate dehydrogenase releasing and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (data not shown).

FFA has multifaceted functions. It is conceivable that the effect of FFA on AMPK activation could be a secondary event resulting from its effects on other target molecules. However,
by using structurally different cyclooxygenase-2 inhibitors and various channel blockers, we excluded this possibility (Supplemental Fig. 1). On the contrary, we believe that the activation of AMPK might be behind the regulatory effects of FFA on these molecules. In support of this notion, implication of AMPK in the inhibition of cyclooxygenase-2 expression has been reported (Lee et al., 2009). In addition, the channels regulated by FFA are, in fact, modulated by AMPK in a similar way. For example, both FFA and AMPK are able to inhibit cystic fibrosis transmembrane conductance regulator and sodium channels (McCarty et al., 1993; Carattino et al., 2005; Kongsuphol et al., 2009; Yau et al., 2010). Moreover, the effective concentrations of FFA used in those studies (1–300 μM) are consistent with the concentrations required for AMPK activation shown in this study.

Activation of AMPK also underlies the anti-inflammatory actions of FFA (Pilon et al., 2004; Cheng et al., 2007; Jeong et al., 2009; Aoki et al., 2010; Cai et al., 2010; Shin et al., 2010). A series of studies demonstrated that AMPK mediates the anti-inflammatory effects of a variety of agents, including nicotine, berberine, cilostazol, and adionepcin (Pilon et al., 2004; Cheng et al., 2007; Jeong et al., 2009; Aoki et al., 2010; Cai et al., 2010). Consistent with these findings, we also found that the suppressive effects of FFA on IL-1β/TNFα-induced NfκB activation and iNOS expression critically depended on the CaMKKβ-AMPK pathway. In addition to iNOS, our preliminary result demonstrated that the cytokine-elicted expression of MCP-1, another NFκB-regulated gene product (Hayakawa et al., 2006b), was suppressed by FFA (Supplemental Fig. 2). Thus the well documented anti-inflammatory effect of FFA could be attributable to AMPK.

FFA has also been documented to be able to inhibit vascular smooth muscle cell proliferation and suppress p44/42-mitogen-activated protein kinase [also known as extracellular signal-related kinase (ERK)] expression (Schöber et al., 2002). It is noteworthy that an antagonistic relationship between AMPK and ERK in the regulation of cell growth and other cell behaviors has been well described previously (Hwang et al., 2006; Du et al., 2008). It is likely that AMPK also contributes to the growth inhibitory action of FFA through suppression of ERK activation.

In addition to unraveling an important molecular mechanism mediating the pharmacological actions of FFA, our findings suggest that the fenamate group of NSAIDs may be used for the treatment of metabolic disorders. As a key regulator of cell metabolism, activation of AMPK underlies the therapeutic benefit of some important anti-inflammatory drugs such as metformin and thiazolidinediones (Towler and Hardie, 2007).

In comparison with the well known AMPK activator AICAR, FFA induced an even rapid and potent activation of AMPK (Supplemental Fig. 3). In addition, cells that are critically involved in lipid and glucose metabolism, such as hepatocytes and preadipocytes, displayed a similar response to FFA. Moreover, FFA also induced AMPK activation in vivo. As a commonly used anti-inflammatory NSAID, FFA might be a promising therapeutic option for treating metabolic diseases, especially for those associated with inflammatory lesions.

In summary, our study indicates that FFA is a potent activator of AMPK. Activation of AMPK could be an important mechanism by which FFA exerts its anti-inflammatory and channel-regulating actions. As a widely used NSAID, FFA may be exploited for the therapeutic intervention of metabolic disorders.

Authorship Contributions

Participants in research design: Chi, Takeda, Kitamura, and Yao.

Conducted experiments: Chi, Li, Yan, Koizumi, Shi, Takahashi, and Zhu.

Contributed new reagents or analytic tools: Koizumi, Matsue, and Takeda.

Performed data analysis: Chi, Zhu, and Yao.

Wrote or contributed to the writing of the manuscript: Chi and Yao.

References


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Nonsteroidal Anti-inflammatory Drug Flufenamic Acid
Is a Potent Activator of AMPK
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Supplemental Fig. 1

Supplemental Figure 1. Effects of COX-2 inhibitors and channel blockers on activation of AMPK. NRK-52E cells were exposed to COX-2 inhibitors FFA (50 μM), indomethacin (100 μM), aspirin (4 mM), and ibuprofen (100 μM; A), or gap junction inhibitor lindane (100 μM), α-glycyrrhetinic acid (10 μM), carbenoxolone (10 μM) and heptanol (3 μM; B), or nonspecific channel inhibitors lanthanum (La$^{3+}$; 1 mM) and gadolinium (Gd$^{3+}$; 500 μM), or TRP channel inhibitor 2-aminoethoxydiphenyl borate (2-APB; 10 μM; C) for 5 min. Cellular proteins were extracted and subjected to Western blot analysis for the phosphorylated AMPKα. β-actin shown at the bottom of the blots indicates the same amount of loading of protein. Results are representatives of two to three separate experiments.
Supplemental Figure 2. Suppression of the cytokines-induced MCP-1 expression by FFA. NRK-52E cells were stimulated with 2 ng/ml IL-1β and 20 ng/ml TNFα in the presence or absence of 50 μM FFA. Cellular proteins were extracted and subjected to Western analysis for MCP-1. Equal loading of protein per lane was verified by probing the blot with an anti-β-actin antibody. Result shown are representative of two separate experiments.
Nonsteroidal Anti-inflammatory Drug Flufenamic Acid Is a Potent Activator of AMPK

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Supplemental Fig. 3

Supplemental Figure 3. Effects of FFA and AICAR on AMPK activation. NRK-E52 cells were exposed to 50 μM FFA (A) or 500 μM AICAR (B) for the indicated time intervals. Cellular protein was extracted and subjected to Western blot analysis using an anti-p-AMPK antibody. The same loading of protein in each lane was verified by probing the blots with an anti-β-actin antibody.