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Nonsteroidal Anti-inflammatory Drug Flufenamic Acid Is a Potent Activator of AMPK

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Running title: **Fenamates activate AMPK**

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ABBREVIATIONS

The abbreviations used are: 2-APB, 2-aminoethoxydiphenyl borate; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CaMKKβ, Ca^{2+}/calmodulin-dependent kinase kinase β; COX-2, cyclooxygenase2; CFTR, cystic fibrosis transmembrane conductance regulator; FFA, flufenamic acid; Gd^{3+}, Gadolinium; La^{3+} , lanthanum; MPTP, mitochondria permeability transition pore; NSAID, nonsteroidal anti-inflammatory drug; Thr-172, threonine-172; TRP, transient receptor potential.

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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 also 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 Thr-172. 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 abolished by treatment of cells with BAPTA-AM (an intracellular Ca^{2+} chelator) or depletion of extracellular Ca^{2+}, whereas it was mimicked by stimulation of cells with Ca^{2+} ionophore A23187 or ionomycin. 3) FFA triggered a rise in intracellular Ca^{2+}, which was abolished by cyclosporine, a blocker of mitochondrial permeability transition pore. Cyclosporine also abolished FFA-induced activation of AMPK. 4) Inhibition of Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ) with STO-609 or downregulation of CaMKKβ with siRNA largely abrogated FFA-induced activation of AMPK. 5) FFA significantly suppressed NF-κB activity and iNOS expression triggered

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by IL-1β and TNFα. This suppression was also largely abrogated by STO-609. Taken together, we conclude that FFA induces AMPK activation through Ca^{2+}-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 threonine-172 (Thr-172) on its α-subunit by upstream kinases LKB-1 and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) (Hawley et al., 2005; Shaw et al., 2004). Once activated, AMPK switches off ATP-consuming pathways, whereas switches on ATP-producing pathways. Besides its regulatory functions on cellular metabolic pathways, AMPK also has anti-inflammatory effects (Aoki et al., 2010; Cai et al., 2010; Cheng et al., 2007; Jeong et al., 2009; Pilon et al., 2004; 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; Klein et al., 2009; Kongsuphol et al., 2009; Kreneisz et al., 2009; Mace et al., 2008).

Flufenamic acid (FFA) is one of the non-steroidal anti-inflammatory drugs (NSAIDs) used for alleviation of inflammation and pains in the clinic (Flower et al., 1972). In

addition, FFA also 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 (CFTR) (McCarty et al., 1993), voltage-gated sodium channels (Yau et al., 2010), transient receptor potential (TRP) channels (Hill et al., 2004) and non-selective cation channels (Poronnik et al., 1992). On another hand, it activates potassium channels (Ottolia and Toro, 1994), TRPC6 (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+} (Gardam et al., 2008; Jordani et al., 2000; McDougall et al., 1988; Poronnik et al., 1992; 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 (Gardam et al., 2008; Poronnik et al., 1992). 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 CaMKKβ pathway (Stahmann et al., 2006). Second, similar to FFA, AMPK has both anti-inflammatory and channel-regulatory activity (Aoki et al., 2010; Cai et al., 2010; Carattino et al., 2005; Cheng et al., 2007; Jeong et al., 2009; Klein et al., 2009; Kongsuphol et al., 2009; Kreneisz et al., 2009; Mace et al., 2008; Pilon et al., 2004; Shin et al., 2010). For example, both FFA and AMPK have been reported to suppress the inflammatory mediators-induced expression of iNOS (Aoki et al., 2010; Paik et al., 2000) 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 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 the evidence showing that FFA potently activates AMPK through Ca^{2+}-CaMKKβ pathway. Activation of AMPK is a presently unrecognized important mechanism underlying the pharmacological actions of FFA.

METHODS

Materials

IL-1β and TNFα were purchased from R&D Systems (Minneapolis, MN, USA). SC-514, aspirin and anti-inducible nitric oxide synthase (anti-iNOS) antibody were obtained from Cayman Chemical Company (MI, USA). Anti-phospho-AMPKα (thr172), anti-phospho-AMPKβ1 (ser108) and anti-phospho-acetyl-coA carboxylase (ACC; ser79) antibodies were obtained from Cell Signaling Inc (Beverly, MA, USA). Fetal bovin serum (FBS), trypsin/EDTA, antibiotics, anti-β-actin antibodies as well as all other chemicals were purchased from Sigma (Tokyo, Japan).

Cells

Normal rat kidney proximal epithelial cells (NRK-52E), porcine kidney epithelial cell line LLC-PK1, mouse hepatoma cell line Hepa 1c1c-7, human epithelial carcinoma cell line (HeLa, PC-3 and LNCaP) and mouse pre-adipocyte cell line 3T3-L1 were purchased from American Type Culture Collection (Rockville, MD). For maintenance, these cells were cultured in DMEM/F-12 containing 5-10% FBS. For experiments, they were cultured in DMEM/F-12 containing 1% FBS.

Animals

Adult female C57BL/6J mice, weighting 25-30 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 (62.5mM Tris-HCl, 2%SDS, 10% glycerol) together 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 r.p.m. for 10 min at 4^0C. Supernatant was recovered and protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Western blot was performed by the enhanced chemiluminescence system (Yao et al., 2005). Briefly, extracted cellular proteins were separated by 10% or 4-20% gradient SDS-polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in PBS, the membranes were incubated with anti-phospho-AMPKα, β, anti-phospho-ACC, or anti-iNOS antibody. After washing, the membranes were probed with horseradish peroxidase-conjugated anti-rabbit IgG, and the bands were visualized by the enhanced chemiluminescence system (Amersham Biosciences, 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^{2+}

Cultured NRK-52E cells were loaded with fura-2 by incubation with 5 μmol/L fura-2 acetoxymethyl ester (Fura-2 AM) in Hanks` balanced salt solution (HBSS) containing 2.0 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ at room temperature. Ca^{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, Japan) at a final concentration of 20 nM using Hyperfect transfection reagent for 48 h. After that, cells were either left untreated or exposed to 50 μM FFA for 5 min. Cellular proteins were extracted and analyzed for the phosphorylated AMPKα.

Establishment of stable transfectant

NRK/NFκB-SEAP reporter cells were established by stably transfection of NRK-52E cells with pNFκB-SEAP (BD Biosciences), as described previously (Hayakawa et al., 2006a; Yao et al., 2005). pNFκB-SEAP encodes SEAP under the control of NFκB.

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 (Hayakawa et al., 2006a; Yao et al., 2005). In brief, five microliters of culture media were mixed with 15 µl of 1x dilution buffer and incubated at 65°C for 30 min. After the incubation, the samples were mixed with 20 µl of assay buffer containing L-homoarginine, left at room temperature for 5 min and added with 20 µl of chemiluminescent enhancer containing 1.25 mM CSPD chemiluminescent substrate. After incubation in the dark for 30 min, the samples were subjected to assays using a luminometer (Gene Light 55; Microtech Nition). All assays were performed in quadruplicate.

Measurement of nitrite levels

NO production was assayed by detecting nitrite accumulation in the culture medium using the Griess reagent (Green et al., 1982). Briefly, 100 µl of a solution containing 1% sulphanilamide, 0.1% naphylethylenediamine 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 NO_2 per microgram of total cellular protein.

Statistical Analysis

Values are expressed as mean ± S.E. Comparison of two populations was made by Student *t*-test. For multiple comparisons with a single control, one-way analysis of variance (ANOVA) followed by Dunnett's test was employed. Both analyses were carried out using the SigmaStat statistical software (Jandel Scientific, CA, USA). $P < 0.05$ was considered to be a statistically significant difference.

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Results

FFA induces AMPK activation

FFA is one of the N-ary-anthranilic acid derives, belonging to fenamate group of NSAID. Other members of fenamate NASIDs include mefenamic acid (MfA), tolfenamic acid (TFA), niflumic acid (NFA) and meclofenamic acid (MCFAA). All these chemicals have similar structure and function (Poronnik et al., 1992; Winder et al., 1963). To test whether fenamates activate AMPK, we examined the influence of these chemicals on phosphorylation level of AMPKα at Thr-172 in NRK-52E cells. Previous studies had established that phosphorylation of this site correlates with AMPK activity (Towler and Hardie, 2007). As shown in Figs. 1A and 1B, incubation of NRK cells with fenamates resulted in increased levels of phosphorylated AMPKα and β, which was associated with paralleled elevation of phosphorylated ACC, one of the AMPK substrate (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 therefore chosen 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 and peaked at about 15 min and retained at a relatively high level for at least 12 h (Figs. 1C and 1D). 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 (Figs. 1E and 1F). These results thus indicate that N-ary-anthranilic acid derivative is a novel class of AMPK activator.

Activation of AMPK by FFA is not the cell type and species-specific

To determine whether the effect of FFA is the cell type and species-specific, we evaluated AMPK activation in several different types of cells. As shown in Fig. 2, FFA caused a concentration-dependent activation of AMPK in pig kidney proximal tubular epithelial cells (LLC-PK1; Fig. 2A), mouse hepatoma cell line Hepa 1c1c-7 cells (Fig. 2B), mouse preadipocyte cell line 3T3-L1 cells (Fig. 2C), human HeLa cells (Fig. 2D) and two human prostate cancer epithelial cells (LNCaP and PC-3; Fig. 2E and 2F, 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 tissues (Fig. 2H). 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 Ca^{2+}

Several studies have demonstrated that FFA is able to elevate intracellular Ca^{2+} through induction of Ca^{2+} releases from mitochondria (Gardam et al., 2008; Jordani et al., 2000; McDougall et al., 1988; Poronnik et al., 1992; Tu et al., 2009). We, therefore, evaluated the role of Ca^{2+} in FFA-induced activation of AMPK. First, we confirmed the calcium-elevating effect of FFA in NRK-52E cells. Consistent with previous reports (Gardam et al., 2008; Jordani et al., 2000; McDougall et al., 1988; Poronnik et al., 1992; Tu et al., 2009), FFA also elevated intracellular Ca^{2+} in NRK-52E cells (Fig. 3). This effect was completely blocked by cyclosporine, an inhibitor of the mitochondria permeability transition pore (MPTP) (Broekemeier and Pfeiffer, 1995).

We then examined the role of the elevated intracellular Ca^{2+} in AMPK activation. As shown in Fig. 4A, induction of intracellular Ca^{2+} with Ca^{2+} inophores A23187 and ionomycin increased phosphorylation levels of AMPKα. In contrast, inhibition of

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intracellular Ca^{2+} by culture of cells in a calcium-free medium or by addition of Ca^{2+} chelator BAPTA-AM largely abrogated AMPK activation (Figs. 4B-4D). Consistent with the causative role of MPTP opening in FFA-induced elevation in intracellular Ca^{2+}, cyclosporine also significantly blocked the AMPK activation (Figs. 4E and 4F). Thus the elevated Ca^{2+} is required for FFA-induced activation of AMPK.

CaMKKβ underlies FFA-induced activation of AMPK

Increased intracellular Ca^{2+} activates various kinases, including a well-documented AMPK kinase CaMKKβ (Hawley et al., 2005). To assess the role of kinases, especially CaMKKβ, we examined the influences of a PKC inhibitor calphostin and a specific CaMKKβ inhibitor STO-609 on the activation of AMPK. As shown in Figs. 5A-5C, both agents effectively suppressed AMPK phosphorylation. Furthermore, downregulation of CaMKKβ 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 also suppress

inflammatory responses (Cheng et al., 2007; Peairs et al., 2009). Therefore, we tested the possible implication of AMPK in the anti-inflammatory effect of FFA. For this purpose, we have examined the proinflammatory cytokines IL-1β and TNFα-induced expression of iNOS. As shown in Fig. 6A and 6B, FFA markedly inhibited the cytokines-induced expression of iNOS in NRK-52E cells. This effect was similarly produced by a well-known AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), suggesting a possible involvement of AMPK. Because activation of AMPK by FFA in NRK-52E cells was mediated by CaMKKβ, we, therefore, determined the role of AMPK by inhibition of CaMKKβ. As shown in Fig. 6C-6E, 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 inhibition of NFκB (Aoki et al., 2010), we, therefore, examined possible effect of FFA on NFκB. First, we confirmed that the cytokines-induced expression of iNOS was controlled by NFκB. Inhibition of NFκB with SC-514 completely abrogated the cytokines-elicited iNOS expression (Fig. 6F). To determine the influence of FFA on NFκB, we have

transfected NRK-52E cells with pNFκB-SEAP and monitored SEAP activity in the conditioned media. As shown in Fig. 6G, FFA significantly inhibited the cytokines-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 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 clinic and basic research, our findings may have significant implications for understanding the pharmacological actions of FFA.

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

al., 2000; McDougall et al., 1988; Poronnik et al., 1992; Tu et al., 2009), this action of FFA was due to the opening of MPTP. Blockade of MPTP with cyclosporine attenuated the increase of Ca^{2+} and abolished the activation of AMPK.

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 (Gardam et al., 2008; Jordani et al., 2000; McDougall et al., 1988; Poronnik et al., 1992; 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. Interestingly, the loss of mitochondria Ca^{2+} has been recognized to be causative of the reduced biogenesis in mitochondria (Cardenas 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 mitochondria.

One previous report demonstrated that PKC mediated the ischemic precondition-induced activation of AMPK (Nishino et al., 2004). Interestingly, 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 due to its inhibition on PKC or intracellular Ca^{2+}. More detailed analysis on this aspect may be needed in the future.

Of note, 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 the concentration as low as 5-10 μM. In contrast, the similar extend 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 up-taking, processing and metabolizing FFA. It could also be due to 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 LDH releasing and MTT 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 COX-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, an implication of AMPK in the inhibition of COX-2 expression has been reported (Lee et al., 2009). In addition, the channels regulated by FFA are, in fact, also modulated by AMPK in a similar way. For example, both FFA and AMPK are able to inhibit CFTR and sodium channels (Carattino et al., 2005; Kongsuphol et al., 2009; McCarty et al., 1993; Yau et al., 2010). Moreover, the effective concentrations of FFA used in the publications (1 to 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 (Aoki et al., 2010; Cai et al., 2010; Cheng et al., 2007; Jeong et al., 2009; Pilon et al., 2004; 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 adiponectin (Aoki et al., 2010; Cai et al., 2010; Cheng et al., 2007; Jeong et al., 2009; Pilon et al., 2004). Consistent with these findings, we also found that the suppressive effects of FFA

on IL-1β/TNFα-induced NFκB activation and iNOS expression were critically dependent on CaMKKβ-AMPK pathway. Besides iNOS, our preliminary result demonstrated that the cytokines-elicited expression of MCP-1, another NFκB-regulated gene product (Hayakawa et al., 2006b), was also 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 suppresses p44/42-mitogen-activated protein kinase (MAPK; also known as extracellular signal-related kinase: ERK) expression (Schober et al., 2002). Interestingly, an antagonistic relationship between AMPK and ERK in regulation of cell growth and other cell behaviors has been well described (Du et al., 2008; Hwang et al., 2006). It is likely that AMPK also contributes to the growth-inhibitory action of FFA through suppression of ERK activation.

Besides unraveling an important molecular mechanism mediating the pharmacological actions of FFA, our findings suggest that fenamates group of NSAIDs may be used for treatment of metabolic disorders. As a key regulator of cell metabolism, activation of

AMPK underlies the therapeutic benefits of some important anti-diabetic drugs like 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 preadepocytes, also 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 to treat 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 therapeutic intervention of metabolic disorders.

Authorship Contributions

Participated in research design: Yao, Chi, Kitamura, Takeda

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

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

Perform data analysis: Chi, Zhu, Yao

Wrote or contribute to the writing of the manuscript: Yao, Chi

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Footnotes

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Legends for Figures

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

Figure 2. Effects of FFA on activation of AMPK in several different cell lines and tissues. (A-D) Effects of FFA on activation of AMPK in several different cell lines. Pig

kidney proximal tubular epithelial cells (LLC-PK1; A), mouse hepatoma cell line Hepa 1c1c-7 cells (B), mouse preadipocyte 3T3-L1 cells (C), human HeLa cells (D), 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 AMPKα at serine 172 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-H) Effects of FFA on AMPK activation *in vivo*. Mice were intraperitoneal injected with the indicated concentrations of FFA for 30 min. Proteins from liver (E) and kidney (F) tissues were extracted and subjected to Western blot analysis for the phosphorylated AMPKα. 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.

Figure 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 (s). The results are presented as dynamic traces of Ca^{2+} over time (A), representing average level of intracellular Ca^{2+} among 15-20 cells in a single study, or intracellular Ca^{2+} level at basal and peak values after 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 *vs*. FFA alone (mean ± SE; n = 15 - 20).

Figure 4. Modulation of intracellular Ca^{2+} on FFA-induced activation of AMPK. (A) Induction of AMPKα phosphorylation by Ca^{2+} inophores A23187 and ionomycin. NRK-52E cells were exposed to 5 μM A23187 and 10 μM ionomycin for 5 min. (B-D) FFA-induced activation of AMPK in the absence of extracellular Ca^{2+} or in the presence of Ca^{2+} chelator BAPTA-AM. NRK-52E cells were either cultured in Ca^{2+}-free medium (B and C) or normal Ca^{2+} medium with 100 μM BAPTA-AM (D) for 1 h before exposing to FFA for additional 5 min. (C) Densitometric analysis of blot shown in Fig. B. Data are expressed as percentage of FFA-stimulated levels of p-AMPK (mean ± SE; n = 3) * P < 0.05 *versus* FFA alone. (E-F) Effects of inhibition of mitochondria permeability transition pore with cyclosporine on FFA-induced activation of AMPK. NRK-52E cells were pretreated with 5 μM cyclosporine for 30 min, and then exposed to 50 μM FFA for 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 Fig. E. Data are expressed as percentage of FFA-stimulated levels of p-AMPK (mean ± SE; n = 3) * P < 0.05 *versus* FFA alone.

Figure 5. Involvement of CaMKKβ in FFA-induced activation of AMPK. (A-C) 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-C) for 15 min, and then exposed to 50 μM FFA for additional 5 min. (C) Densitometric analysis of blot shown in Fig. B. Data are expressed as percentage of FFA-stimulated levels of p-AMPK (mean ± SE; 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.

Figure 6. Suppressive effects of FFA on the cytokines-induced iNOS expression and NFκB activation. (A-B) Suppression of the cytokines-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. Result was expressed as percentage of the cytokines-stimulated level of iNOS (mean ± SE; n = 3), * $P < 0.05$ *versus* the cytokines-stimulated cells. (C-D) Attenuation of FFA-induced suppression of iNOS by inhibition of AMPK kinase CaMKKβ. 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. Result was expressed as percentage of the cytokines-stimulated level of iNOS (mean ± SE; n = 3), * $P < 0.05$. NS = not significantly different ($P > 0.05$). (E) Abrogation of FFA-induced suppression of NO formation by inhibition of AMPK kinase CaMKKβ. NRK-52E cells were treated as D. The conditioned media were harvested for measurement of nitrite levels. Data are expressed as mean ± SE; n = 4. * $P < 0.05$. NS = not significantly different ($P > 0.05$). (F) Role of NFκB in the cytokines-induced iNOS expression. NRK cells were treated with 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 by 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 AMPK kinase CaMKKβ. 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 ± SE (n = 4). * $P < 0.05$. NS = not significantly different ($P > 0.05$).

Figure 7. Schematic diagram illustrating potential mechanisms involved in FFA-induced activation of AMPK and suppression of iNOS. FFA elevates intracellular Ca^{2+} through induction of the release of Ca^{2+} from mitochondria, causing activation of CaMKKβ-AMPK pathway. The activated AMPK contributes to the inhibition of NFκB and NFκB-regulated iNOS expression.

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Fig 1

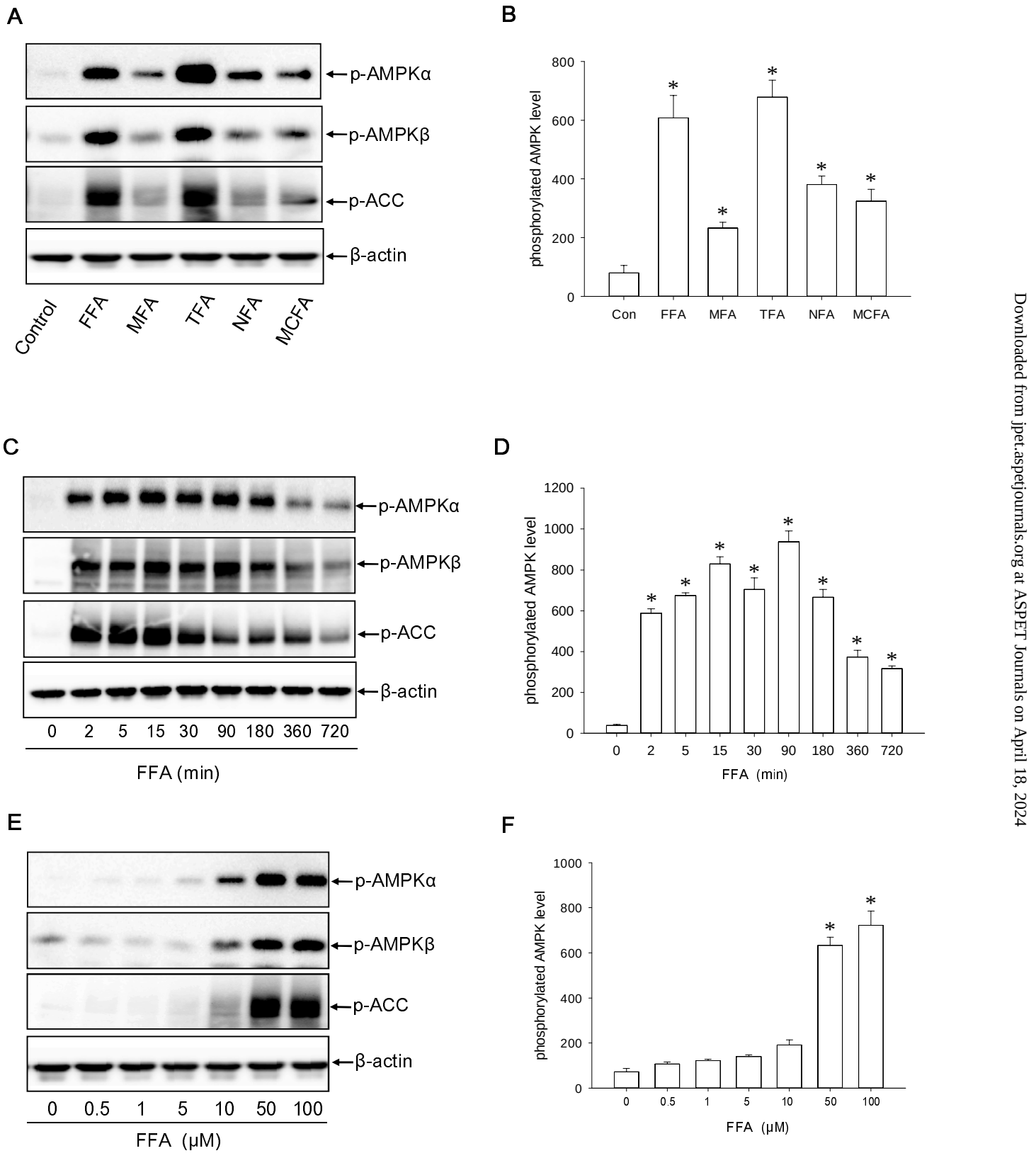

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Fig 2

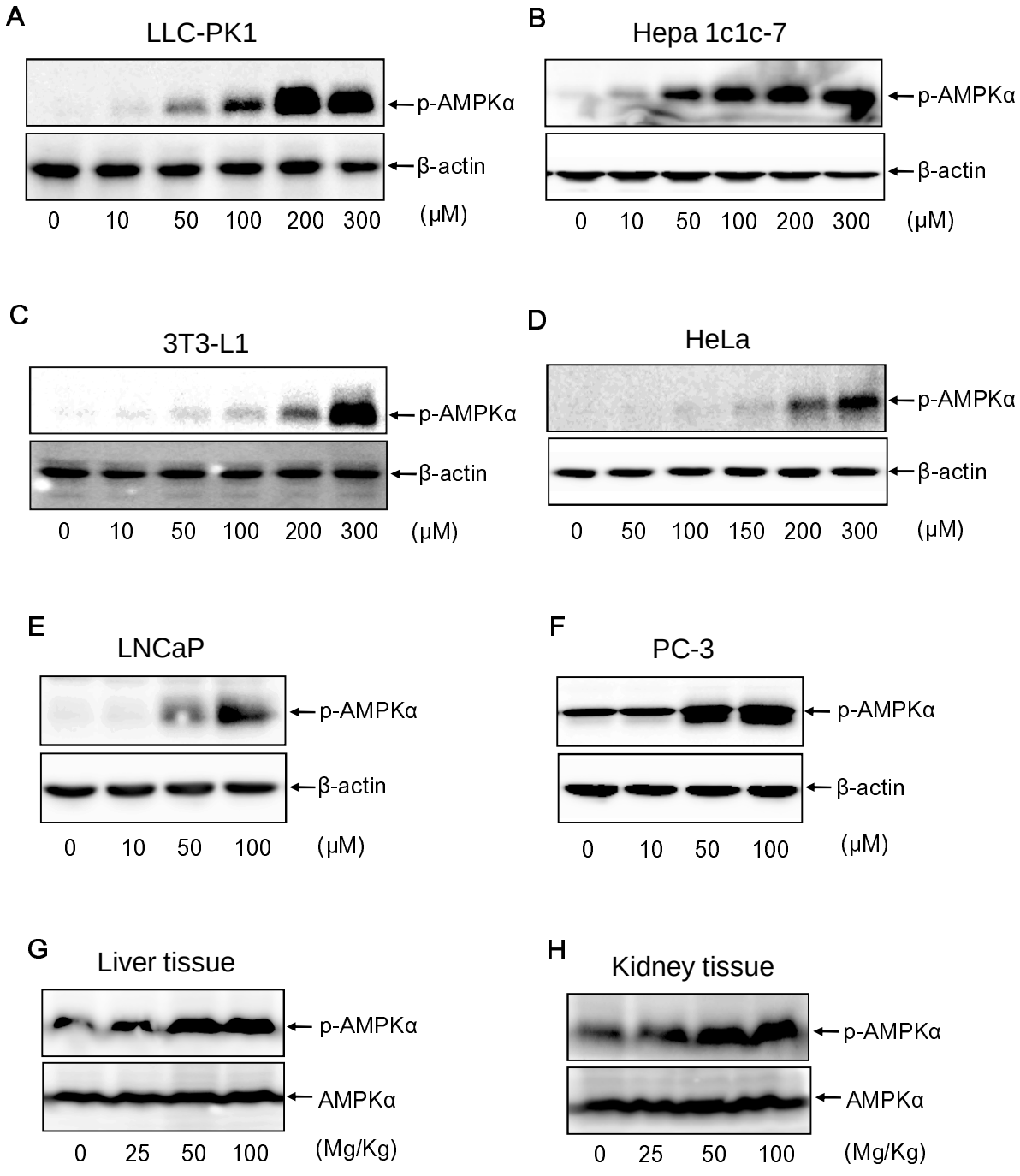

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Fig 3

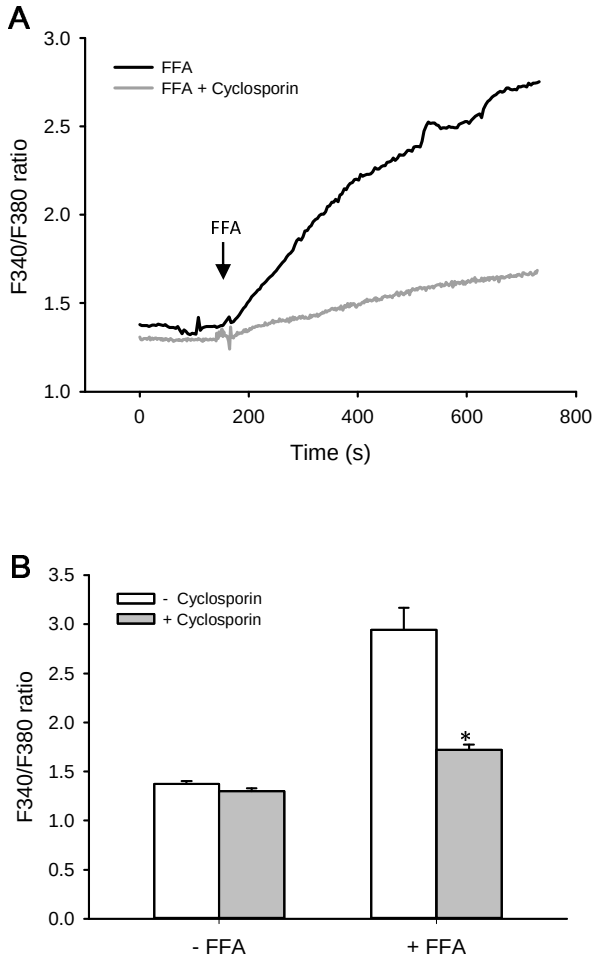

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Fig 4

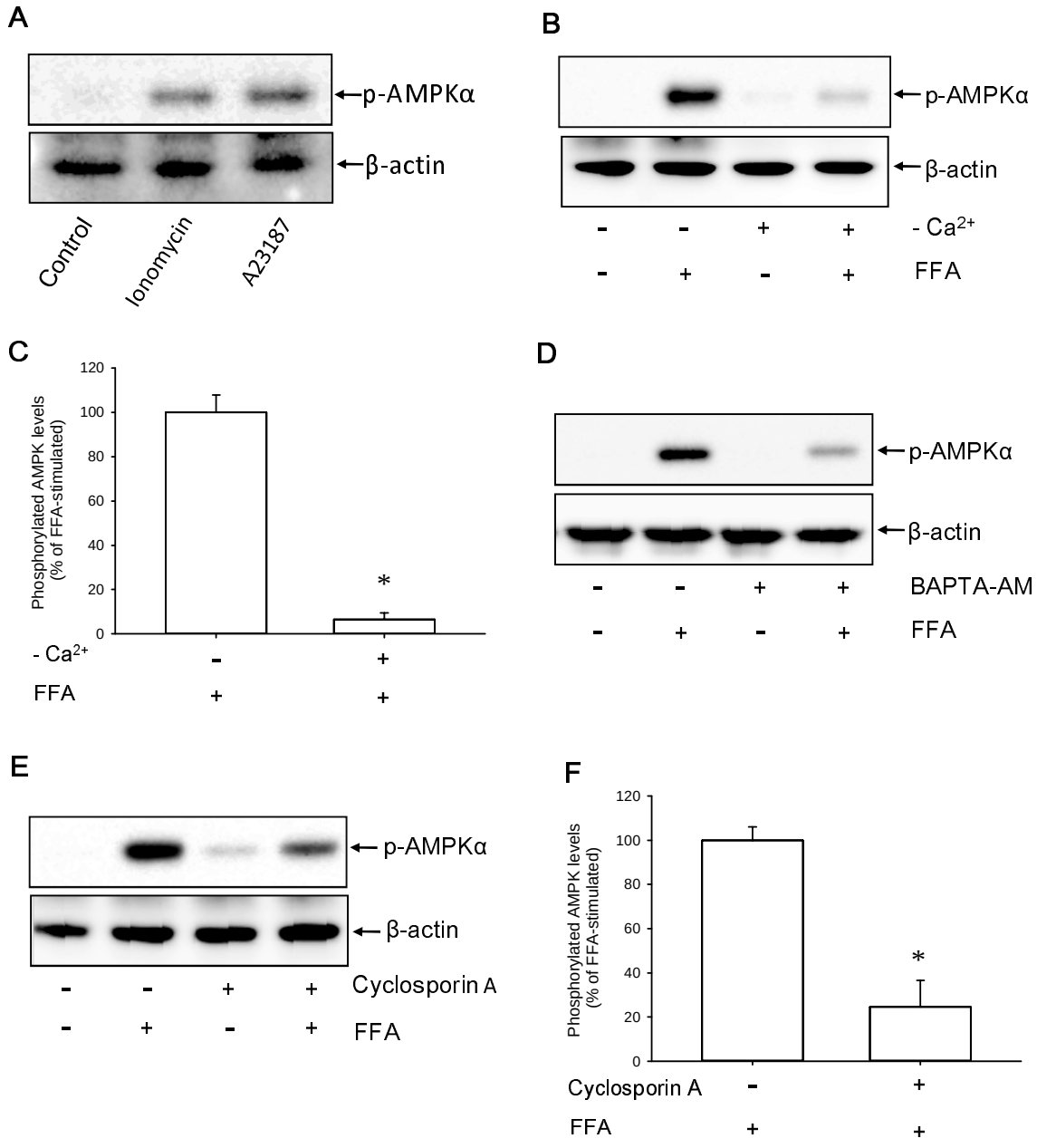

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Fig 5

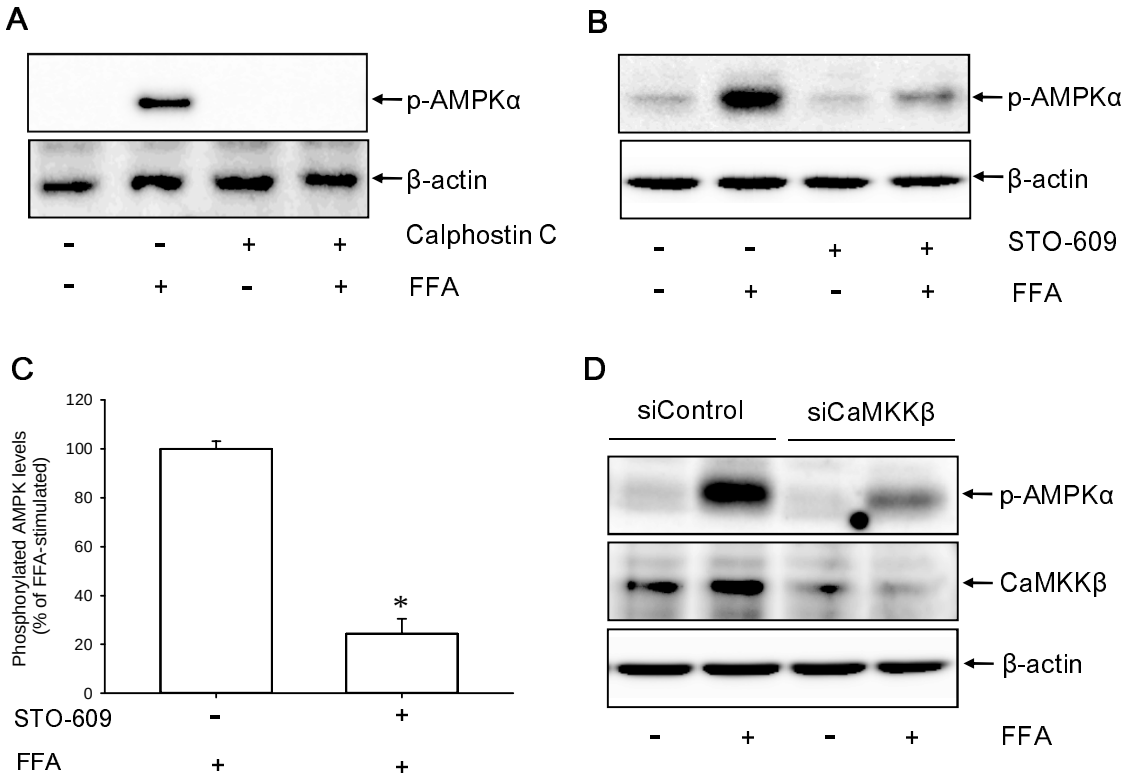

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Fig 6

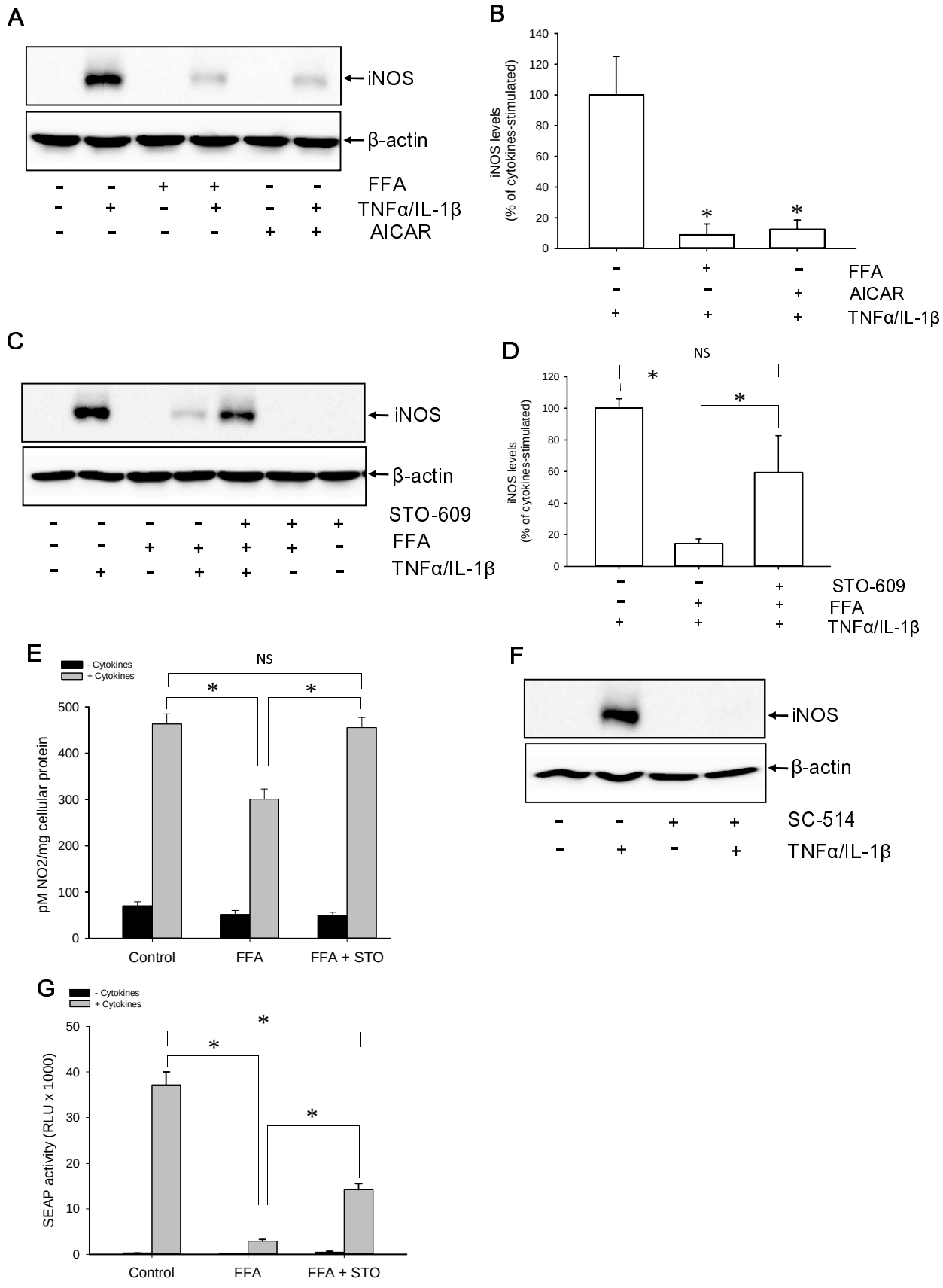

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Fig 7