Oxidative stress induces cell death partially by decreasing both mRNA and protein levels of nicotinamide phosphoribosyltransferase in differentiated PC12 cells

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Background. Multiple studies have indicated crucial roles of NAD^+ deficiency in several neurological diseases and aging. It is critical to discover the mechanisms underlying the NAD^+ deficiency. Decreased level of Nicotinamide phosphoribosyltransferase (Nampt) - an important enzyme in the salvage pathway of NAD^+ synthesis - has been found under certain pathological conditions, while the mechanisms underlying the Nampt decrease are unclear. The purpose of this study is to test the hypothesis that oxidative stress can produce decreased Nampt, and to investigate the biological effects of Nampt on NAD^+ synthesis and cell survival under both basal and oxidative stress conditions.

Methods. We used differentiated PC12 cells as a cellular model to investigate the effects of oxidative stress on the levels of Nampt. Multiple assays, including flow cytometry-based cell death assays and NAD^+ assays were conducted.

Results. First, oxidative stress can decrease the levels of Nampt mRNA and Nampt protein; second, Nampt plays significant roles in NAD^+ synthesis under both basal conditions and oxidative stress conditions; third, Nampt plays critical roles in cell survival under both basal conditions and oxidative stress conditions; and fourth, oxidative stress produced decreased NAD^+ levels and cell survival partially by decreasing Nampt. Collectively, our study has indicated that oxidative stress is a pathological factor leading to decreased Nampt, which plays important roles in oxidative stress-produced decreases in NAD^+ levels and cell survival. Our findings have indicated major roles of Nampt in maintaining NAD^+ levels and cell survival under both basal and oxidative stress conditions.

1 **Oxidative stress induces cell death partially by decreasing both mRNA and protein levels of**

2 **nicotinamide phosphoribosyltransferase**

3 **in differentiated PC12 cells**

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Abstract

24   **Background.** Multiple studies have indicated crucial roles of NAD^+ deficiency in several

25   neurological diseases and aging. It is critical to discover the mechanisms underlying the NAD^+

26   deficiency. Decreased level of Nicotinamide phosphoribosyltransferase (Nampt) - an important

27   enzyme in the salvage pathway of NAD^+ synthesis - has been found under certain pathological

28   conditions, while the mechanisms underlying the Nampt decrease are unclear. The purpose of this

29   study is to test the hypothesis that oxidative stress can produce decreased Nampt, and to investigate

30   the biological effects of Nampt on NAD^+ synthesis and cell survival under both basal and oxidative

31   stress conditions.

32   **Methods.** We used differentiated PC12 cells as a cellular model to investigate the effects of

33   oxidative stress on the levels of Nampt. Multiple assays, including flow cytometry-based cell death

34   assays and NAD^+ assays were conducted.

35   **Results.** First, oxidative stress can decrease the levels of Nampt mRNA and Nampt protein;

36   second, Nampt plays significant roles in NAD^+ synthesis under both basal conditions and oxidative

37   stress conditions; third, Nampt plays critical roles in cell survival under both basal conditions and

38   oxidative stress conditions; and fourth, oxidative stress produced decreased NAD^+ levels and cell

39   survival partially by decreasing Nampt. Collectively, our study has indicated that oxidative stress

40   is a pathological factor leading to decreased Nampt, which plays important roles in oxidative

41   stress-produced decreases in NAD^+ levels and cell survival. Our findings have indicated major

42   roles of Nampt in maintaining NAD^+ levels and cell survival under both basal and oxidative stress

43   conditions.

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45   ***Keywords***: Nicotinamide phosphoribosyltransferase; NAD$^+$; Oxidative Stress; Cell Death; Aging.

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62 **Introduction**

63   Cumulative evidence has domonstrated that NAD^+ plays significant roles in multiple

64 biological processes (Ying 2008). Multiple studies have also suggested that NAD^+ deficiency is a

65 pivotal pathological factor in numerous major diseases and pathological conditions (Ying 2008;

66 Ying 2006; Zhang & Ying 2019), including ischemic brain injury (Ying et al. 2007; Zheng et al.

67 2012), myocardial ischemia (Zhang et al. 2016), head trauma (Won et al. 2012), epilepsy (Liu et

68 al. 2017), radiation-induced tissue injury (Sheng et al. 2012) and chemotherapy agent-induced

69 tissue injury (Wang et al. 2014). NAD^+ deficiency also appears to play an important role in the

70 aging process (Frederick et al. 2016; Mills et al. 2016; Son et al. 2016), which has been the

71 biological basis for the wide applications of NAD^+ and NAD^+ precursors for delaying aging. It has

72 critical theoretical and medical importance to expose the impact of NAD^+ metabolism on

73 pathological alterations. However, there have been no sufficient studies on this critical topic.

74   The salvage pathway and the *de novo* pathway are two major pathways for NAD^+ synthesis

75 (Magni et al. 2004). The nuclear enzyme Nicotinamide mononucleotide adenylyltransferase-1

76 (NMNAT-1) is a crucial enzyme in the two pathway of NAD^+ synthesis (Magni et al. 2004).

77 Mammals use nicotinamide as the precursor for NAD^+ synthesis in the salvage pathway (Revollo

78 et al. 2007): Nicotinamide phosphoribosyltransferase (Nampt) converts nicotinamide to

79 nicotinamide mononucleotide that can be converted to NAD^+ by NMNATs (Revollo et al. 2007).

80   NAD^+ deficiency has been observed in a variety of disease models, which may result from

81 poly (ADP-ribose) polymerase-1 activation or reduced Nampt activity. It has been indicated that

82 decreased Nampt activity is an important mechanism accounting for declined NAD^+ synthesis:

83 Decreased Nampt activity has been found in the metabolic organs that are exposed to high-fat diet

84 (Yoshino et al. 2011), Drosophila pink1 mutants (Lehmann et al. 2017), and aging (Jokinen et al.

85 2017; Yoshino et al. 2011). It is necessary to investigate the potential mechanisms for the

86 decreased Nampt levels.

87   Our study used PC12 cells to investigate the effects of oxidative stress on both the Nampt

88 mRNA and Nampt protein levels. We also investigated the roles of Nampt in the cell death and

89 NAD$^+$ metabolism under both basal and oxidative stress conditions. Our experimental results have

90 shown that oxidative stress can produce decreased levels of Nampt, which contributes to oxidative

91 stress-produced decreases in NAD$^+$ levels and cell survival. Our current study has also indicated

92 that Nampt plays important roles in maintaining intracellular NAD$^+$ levels and cell survival under

93 both basal and oxidative stress conditions.

94

95 **Materials and methods**

96 **Materials**

97   Sigma Aldrich (St Louis, Missouri, USA) was the supplier of H$_2$O$_2$ (323381) and

98 Nicotinamide (72340). FK866 (HY-50876) and P7C3 (HY-15976) were purchased from

99 MedChemExpress (New Jersey, USA). Nampt siRNAs and control siRNAs were purchased

100 from GenePharma (Shanghai, China). Sangon Biotech (Shanghai, China) was the supplier of

101 Nampt primers.

102

103 **Cell cultures**

104 We purchased differentiated PC12 cells from the Cell Resource Center of Shanghai

105 Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). PC12 cells

106 were plated into 12- well or 24-well cell culture plates in DMEM (Hyclone, Massachusetts,

107 USA) containing 10% FBS, 1% penicillin and 1% streptomycin. The cells were cultured in an

108 incubator with 5% CO_2 and the culture temperature was 37°C.

109

110 **NAD^+ assay**

111   As previously described (Alano et al. 2004), recycling assay was conducted to determine

112 the level of intracellular NAD^+:   Cells were lysed by perchloric acid. Samples were

113 centrifugated at 12,000 g for 5 minutes, and the supernatant was transferred to new test tubes.

114 3 N potassium hydroxide and 1 M potassium phosphate buffer were used to neutralize the

115 samples to pH 7.2. After centrifugation, the samples were mixed with reaction buffer that

116 contained 1.7 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT),

117 488.4 mg nicotinamide, 10.6 mg phenazine methosulfate,1.3 mg alcohol dehydrogenase, and

118 2.4 mL ethanol in 37.6 mL Gly-Gly buffer (65 mM, pH 7.4). Ten minutes later, the A_{560nm} of

119 the samples was assessed. The final concentrations of NAD^+ were normalized by protein levels.

120

121 **Annexin V/7-AAD assay**

122 Flow cytometry assay was conducted to determine the levels of necrosis and apoptosis of

123 PC12 cells, and the ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) was

124 used. Briefly, differentiated PC12 cells were harvested by 0.25% trypsin solution. After washes

125 with PBS, the cells were incubated with 100 μL cold 1X binding buffer and 5 μL Annexin V,

126 which were put in ice for 15 minutes. Finally, 200 μL 1X binding buffer and 5 μL 7-AAD were

127 mixed into the samples. The levels of necrosis and apoptosis of PC12 cells were measured by

128 a flow cytometer (FACSAria II, BD Biosciences).

129

130 **Real-Time PCR assay**

131 The total RNA of PC12 cells was harvested by TaKaRa RNA Extraction Kit (Takara

132 Bio, Dalian, China) according to the manufacturer's protocol. cDNA was reverse-transcribed

133 from 500 ng total RNA using a Prime-Script RT reagent kit produced by Takara Bio (Dalian,

134 China). The reverse transcription was conducted as follows: 37°C for 15 minutes, followed by

135 85°C for 15 seconds. SYBR Premix Ex Taq produced by Takara Bio (Dalian, China) was used

136 for conducting Quantitative RT-PCR, in which the following primers were used: Nampt (sense

137 5'- TATTCTGTTCCAGCGGCAGA -3' and anti-sense 5'-

138 GACCACAGACACAGGCACTGA -3') ; GAPDH (sense 5'-

139 CCTGCACCACCAACTGCTTA -3' and anti-sense 5'- GGCCATCCACAGTCTTCTGA -3').

140 The following procedure was conducted for the assays: After denaturation at 95 °C for 10

141 seconds, 40 cycles of 95 °C for 5 seconds and 60° C for 30 seconds were conducted. The

142 comparative threshold cycle method was used for the data analyses. The results were

143 normalized by GAPDH mRNA level.

144

145 **Western blot assay**

146 After treatment, PC12 cells were washed twice with PBS. The cells were lysed in RIPA

147 buffer produced by Millipore (Temecula, California, USA) which contained 1 mM

148 phenylmethanesulfonyl fluoride and 1% protease inhibitor cocktail produced by CWBio

149 (Beijing, China). After centrifugation at 12,000 rpm for 10 minutes at 4 °C, the protein

150 concentrations of the samples were determined by BCA Protein Assay Kit (Pierce

151 Biotechnology, Rockford, Illinois, USA). Thirty μg of the sample was electrophoresed through

152 a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to 0.45-μm

153 nitrocellulose membranes. The blots were incubated with a polyclonal Nampt antibody from

154 Abcam (Cambridge, UK), which was diluted in TBST containing 1% bovine serum albumin at

155 1:2000 ratio, at 4°C overnight. Subsequently, a horse radish peroxidase-conjugated secondary

156 antibody produced by Epitomics (Zhejiang Province, China) diluted by TBST containing 1%

157 bovine serum albumin at 1:3000 ratio was used for incubation of the blots for 1 hr at room

158 temperature. The normalization of the sample was conducted by using a Tubulin antibody

159 produced by proteintech (Wuhan, China). Quantifications of the bands were performed by Gel-

160 Pro Analyzer (Media Cybernetics, Silver Spring, Maryland, USA).

161

162 **RNA interference**

163 PC12 cells at the density of approximately 40% were transfected with he scrambled

164 control siRNA sequences and Nampt siRNA with the following sequences: Sense 5'-

165 AGUAAGGAAGGUGAAAUACTT-3' and antisense 5'-

166 GUAUUUCACCUUCCUUACUTT-3'. The transfection reagent used was lipofectamine 2000

167   (Invitrogen, Carlsbad, California, USA). Each well contained 2.5 µl lipofectamine 2000, 0.06

168   nmol siRNA sequences,100 µl Opti-MEM, and 500 µl Dulbecco's modified Eagle medium.

169   After 6 hrs, the media was replaced by Dulbecco's modified Eagle medium containing 10%

170   FBS.

171

172   **Statistical analyses**

173   Data were shown as mean $\pm$ SEM, which were analyzed by one-way ANOVA or two-way

174   ANOVA. Student - Newman - Keuls *post hoc* tests were used.

175

176   **Results**

177   **1. Nampt inhibitor FK866 significantly decreased the intracellular NAD$^+$ level and cell**

178   **survival of PC12 cells under basal conditions**

179   We investigated the roles of Nampt in maintaining the NAD$^+$ level of PC12 cells under

180   basal conditions. Nampt inhibitor FK866 was used to attenuate the activity of Nampt.   Our

181   experimental results showed that FK866 at the concentrations of 2, 5, 10, 20, and 50 nM produced

182   dose-dependent decreases in the NAD$^+$ levels (Fig. 1A), indicating a critical role of Nampt in the

183   NAD$^+$ synthesis under basal conditions. We also conducted FACS-based Annexin V/7-ADD assay

184   to evaluate the role of Nampt in the cell survival under basal conditions, showing that FK866

185   produced dose-dependent increases in cell death, which equals to the sum of early-stage apoptosis,

186   late-stage apoptosis and necrosis (Figs. 1B and 1C).

187

2. H₂O₂ produced significant decreases in both Nampt mRNA levels and Nampt protein

levels of PC12 cells

We further determined the impact of oxidative stress on the levels of Nampt mRNA and

Nampt protein of PC12 cells: Treatment of the cells with 0.1 or 0.3 mM H_2O_2 led to significant

increases in the Nampt mRNA level of the cells 12 hrs after the treatment (Fig. 2A). In contrast,

H_2O_2 led to decreases in the Nampt mRNA level 20 hrs after the treatment (Fig. 2A). H_2O_2 did not

significantly affect the protein level of Nampt 12 hrs after the H_2O_2 treatment (Figs. 2B and 2E),

while H_2O_2 led to significant decreases in the Nampt protein level both 24 hrs (Figs. 2C and 2E)

and 48 hrs (Figs. 2D and 2E) after the H_2O_2 treatment.

3. Decreased Nampt led to further decreases in the H_2O_2-produced reductions of the NAD^+

levels and survival of PC12 cells

To investigate the biological consequences of the H_2O_2-produced decreases in the Nampt

levels of PC12 cells, we applied both Nampt siRNA and FK866 to determine whether the

decreased Nampt affect the intracellular NAD^+ levels and cell survival of the PC12 cells exposed

to H_2O_2. The results showed that Nampt siRNA treatment led to a marked decrease in the Nampt

level (Figs. 3A and 3B). Nampt siRNA treatment also significantly reduced the intracellular NAD^+

level of PC12 cells under basal conditions (Fig. 3C). Moreover, the Nampt siRNA treatment

further reduced the intracellular NAD^+ level that was decreased by H_2O_2 treatment (Fig. 3C). Our

results also showed that the Nampt siRNA treatment produced increased cell death under basal

conditions (Figs. 3D and 3E). H_2O_2 treatment also increased the cell death, which was further

209   enhanced by the Nampt siRNA treatment (Figs. 3D and 3E).

210   We found that FK866 further reduced the intracellular NAD^+ level that was decreased by

211   H_2O_2 treatment (Fig. 3F). Moreover, the FK866 treatment further enhanced the levels of the H_2O_2-

212   produced necrosis, late-stage apoptosis and early-stage apoptosis (Figs. 3G and 3H).

213

214   **4. Nampt activator P7C3 suppressed the H_2O_2-produced decreases in the NAD^+ levels and**

215   **cell survival of PC12 cells**

216   To further investigate the biological consequences of the H_2O_2-produced reduction of the

217   Nampt protein levels of PC12 cells, we used Nampt activator P7C3 to determine the roles of Nampt

218   activation in the intracellular NAD^+ levels and survival of PC12 cells exposed to H_2O_2: P7C3

219   attenuated the H_2O_2-produced decrease in the intracellular NAD^+ level of PC12 cells (Fig. 4A).

220   P7C3 also reduced the H_2O_2-produced increase in the cell death (Figs. 4B and 4C).

221   We also determined the effects of nicotinamide (NAM) - a substrate of Nampt - on both

222   intracellular NAD^+ levels and cell death of H_2O_2-treated PC12 cells. NAM attenuated the H_2O_2-

223   produced decrease in the intracellular NAD^+ level (Supplemental Fig. 2A). NAM also attenuated

224   the H_2O_2-produced increase in the cell death (Supplemental Figs. 2B and 2C).

225

226   **Discussion**

227   Our study has generated the following important findings: First, Nampt plays critical roles

228   in both NAD^+ synthesis and survival of PC12 cells under basal conditions; second, H_2O_2 produced

229   significant decreases in both Nampt mRNA levels and Nampt protein levels of PC12 cells; and

230   third, H_2O_2 induced cell death partially by producing the decreases in both Nampt mRNA levels

231   and Nampt protein levels, since the Nampt siRNA and Nampt inhibitor significantly exacerbated

232   the H_2O_2-produced decreases in the intracellular NAD^+ level and cell survival, while the Nampt

233   activator attenuated the H_2O_2-produced decreases in the intracellular NAD^+ level and cell survival.

234   Cumulating evidence has suggested crucial roles of impaired NAD^+ metabolism in both

235   aging and multiple diseases (Ying 2008; Ying et al. 2007; Zhang & Ying 2019), indicating that it

236   is necessary to determine the roles of NAD^+ deficiency under these conditions (Ying 2008; Ying

237   et al. 2007; Zhang & Ying 2019). In the tissues or organs under certain pathological conditions,

238   including the metabolic organs that are exposed to HFD (Yoshino et al. 2011), Drosophila pink1

239   mutants (Lehmann et al. 2017), and aging (Jokinen et al. 2017; Yoshino et al. 2011), decreased

240   levels of Nampt have been reported. However, there is significant deficiency in the mechanisms

241   underlying the Nampt alterations under pathological conditions.

242   Our current study has shown that H_2O_2 was capable of decreasing both protein levels and

243   mRNA levels of Nampt in PC12 cells. Increased oxidative stress has been found in aging and

244   multiple pathological conditions (Rani et al. 2016; Ureshino et al. 2014; Zuo et al. 2019).

245   Therefore, our finding has suggested a novel mechanism regarding the Nampt decrease in aging

246   and under multiple pathological conditions. Due to the crucial roles of NAD^+ deficiency in aging

247   and numerous diseases, our finding has also suggested a novel relationship between the

248   pathological alterations and NAD^+ metabolism in aging and the diseases: The oxidative stress in

249   the diseases and aging led to decreased Nampt, producing decreased NAD^+ synthesis thus resulting

250   in multiple pathological changes.

251   Our study found that oxidative stress produced biphasic changes of Nampt mRNA levels

252   and Nampt protein levels, which is reasonable for the following reasons: Since NAD^+ was involved

253   in a variety of biological processes, oxidative stress-produced NAD^+ decreases can lead to cell

254   death. For defensing oxidative stress, both Nampt mRNA levels and Nampt protein levels can be

255   induced by oxidative stress in the early phase. When the oxidative stress last for a significant

256   duration of time, cells may lose the capacity to maintain the Nampt mRNA levels and Nampt

257   protein levels, leading to reductions of the mRNA and protein levels of Nampt.

258   Our current findings have provided evidence that Nampt plays an important role in

259   oxidative stress-induced cell death: Both the Nampt inhibitor and the Nampt siRNA significantly

260   exacerbated the H_2O_2-produced reductions of the NAD^+ level and cell survival, while the Nampt

261   activator significantly attenuated the H_2O_2-induced reductions of the NAD^+ level and cell survival.

262   These observations have suggested a mechanism regarding oxidative stress-produced cell death at

263   least for some cell types.

264   There are two NAD^+ synthesis pathways in cells, while the effects of Nampt on the general

265   NAD^+-generating ability and survival of various cell types under basal conditions are unclear. Our

266   current findings have indicated that Nampt plays critical roles in both NAD^+ synthesis and cell

267   survival under basal conditions in differentiated PC12 cells, since the Nampt siRNA and Nampt

268   inhibitor FK866 produced profound decreases in both NAD^+ levels and survival of the cells under

269   basal conditions.

270

271   **Conclusion**

272   Our study has indicated that oxidative stress is capable of producing decreased Nampt. Our

273 study has also suggested that Nampt plays important roles in maintaining both intracellular NAD^+

274 levels and cell survival under basal conditions and oxidative stress conditions.

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Figure 1

Fig. 1. Nampt plays a siginificant role in the NAD$^+$ synthesis and basal survival of differentiated PC12 cells.

(A) FK866 at the concentrations of 2, 5, 10, 20, and 50 nM produced dose-dependent

decreases in the intracellular NAD$^+$ levels. NAD$^+$ assays were conducted 6 hrs after the cells

were treated with 2, 5, 10, 20, or 50 nM FK866. The data were pooled from three

independent experiments. $N = 3$. There were 3, 3 and 3 samples in these three independent

experiments. ✳✳✳, $P < 0.001$. (B,C) Ten nM FK866 led to a significant increase in early-stage

apoptosis, while both 20 nM and 50 nM FK866 led to significant increases in early-stage

apoptosis (Annexin V$^+$/7-ADD$^-$), late-stage apoptosis (Annexin V$^+$/7-ADD$^+$) and necrosis

(Annexin V$^-$/7-ADD$^+$). PC12 cells were treated with 10, 20, or 50 nM FK866 for 24 hrs. The

data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 3

samples in these three independent experiments. ✳✳, $P < 0.01$; ✳✳✳, $P < 0.001$.
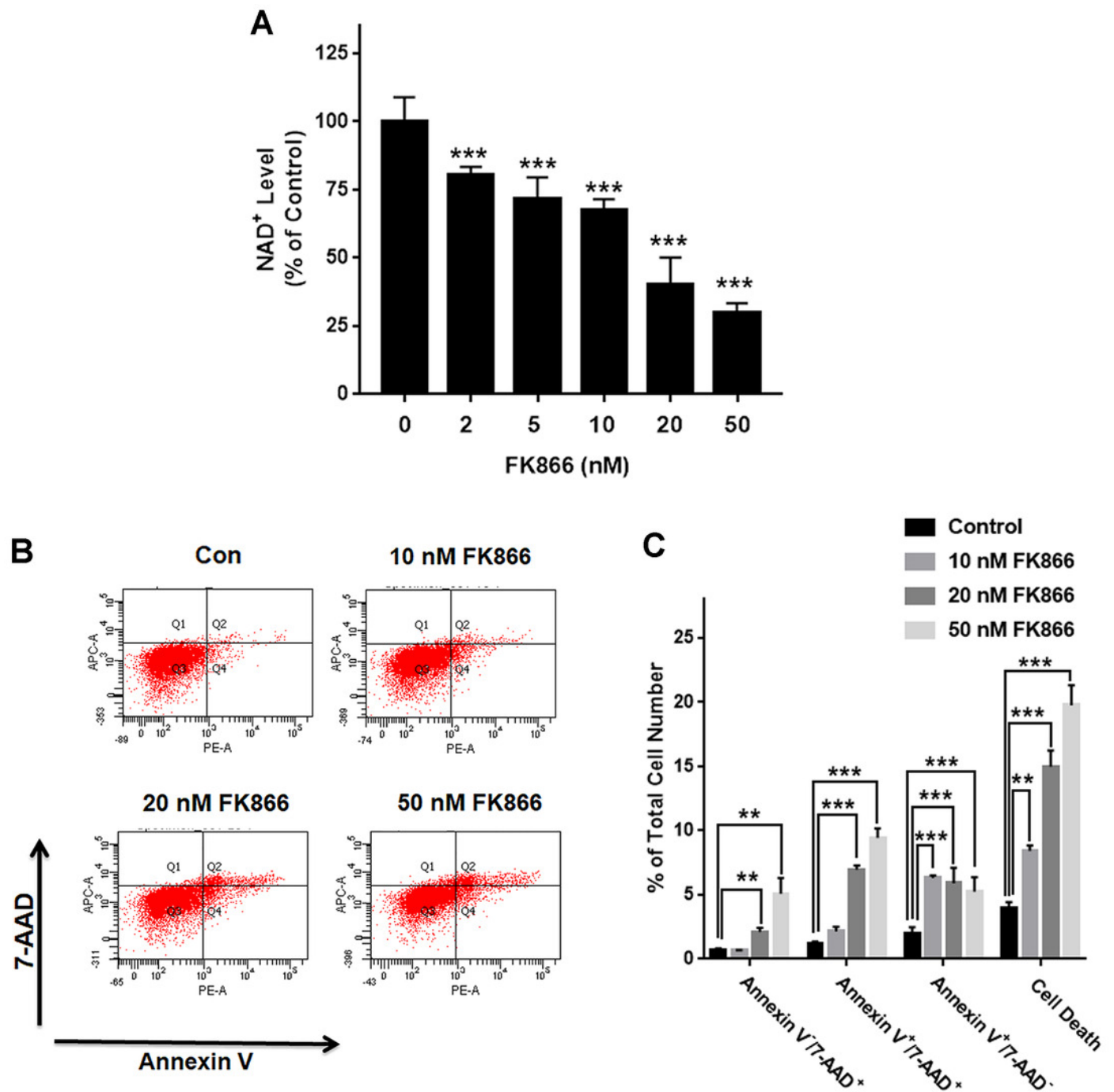
Figure 2

Fig. 2. H_2O_2 produced significant decreases in both Nampt mRNA levels and Nampt protein levels of PC12 cells.

(A) Treatment of PC12 cells with 0.1 mM or 0.3 mM H_2O_2 led to significant increases in the Nampt mRNA level of the cells 12 hrs after the H_2O_2 treatment. In contrast, H_2O_2 led to significant decreases in the Nampt mRNA level of the cells 20 hrs after the H_2O_2 treatment. PC12 cells were treated with 0.1 or 0.3 mM H_2O_2 for 12 or 20 hrs. The data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 3 samples in these three independent experiments. $*$, $P < 0.05$; $**$, $P < 0.01$. (B) Western blot assays showed the e ffects of H_2O_2 on the protein level of Nampt 12 hrs after the H_2O_2 treatment. (C) Western blot assays showed the effects of H_2O_2 on the protein level of Nampt 24 hrs after the H_2O_2 treatment. (D) Western blot assays showed the effects of H_2O_2 on the protein level of Nampt 48 hrs after the H_2O_2 treatment. (E) Quantifications of the Western blot assays showed that H_2O_2 led to significant decreases in the Nampt protein level of the cells 24 and 48 hrs, but not 12 hrs, after the H_2O_2 treatment. PC12 cells were treated with 0.1 or 0.3 mM H_2O_2 for 12, 24, or 48 hrs. The data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 6 samples in these three independent experiments. $*$, $P < 0.05$; $**$, $P < 0.01$.

A

Nampt mRNA Expression

- ■ Control
- ■ 0.1 mM H_2O_2
- ■ 0.3 mM H_2O_2

(12 hrs, 20 hrs)

B H_2O_2 (mM)   0   0.1   0.3

Nampt

Tubulin   12 hrs

C H_2O_2 (mM)   0   0.1   0.3

Nampt

Tubulin   24 hrs

D H_2O_2 (mM)   0   0.1   0.3

Nampt

Tubulin   48 hrs

E

Nampt Protein Level (% of Control)

- ■ Control
- ■ 0.1 mM H_2O_2
- ■ 0.3 mM H_2O_2

(12 hrs, 24 hrs, 48 hrs)
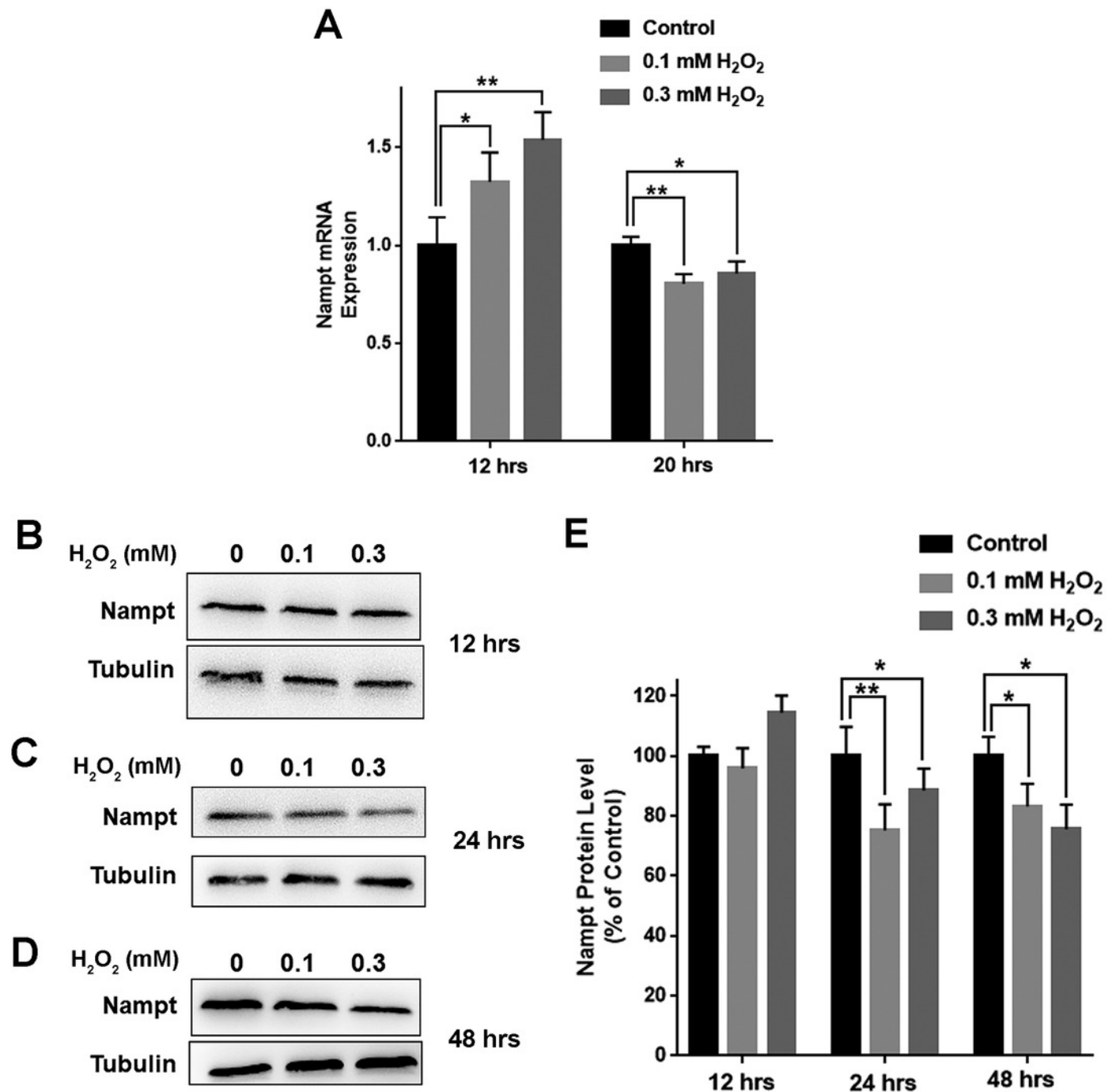
Figure 3

Fig. 3. Nampt siRNA and FK866 significantly exacerbated H_2O_2-produced decreases in the intracellular NAD^+ levels and cell survival of differentiated PC12 cells.

(A, B) Treatment of the cells with Nampt siRNA led to a significant decrease in the Nampt protein level. PC12 cells were transfected with control or Nampt siRNA sequences for 48 hrs. There were 12 samples in the experiments. (C) Nampt siRNA treatment significantly exacerbated H_2O_2-produced decrease in the NAD^+ levels of PC12 cells. (D, E) Nampt siRNA significantly exacerbated H_2O_2-produced late-stage apoptosis (Annexin V^+/7-ADD$^+$) and necrosis (Annexin V^-/7-ADD$^+$) of the cells. PC12 cells were transfected with control or Nampt siRNA sequences for 24 hrs, and then co-treated with 0.3 mM H_2O_2 for 6 hrs (for NAD^+ assay) or 24 hrs (for flow cytometry assay). The data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 3 samples in these three independent experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$. (F) FK866 significantly exacerbated H_2O_2-produced decrease in the NAD^+ levels of PC12 cells. (G, H) FK866 significantly exacerbated H_2O_2-produced early-stage apoptosis (Annexin V^+/7-ADD$^-$), late-stage apoptosis (Annexin V^+/7-ADD$^+$) and necrosis (Annexin V^-/7-ADD$^+$) of the cells. PC12 cells were pre-treated with 10 nM FK866 for 30 min, and then co-treated with 0.3 mM H_2O_2 for 6 hrs (for NAD^+ assay) or 23.5 hrs (for flow cytometry assay). The data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 3 samples in these three independent experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.
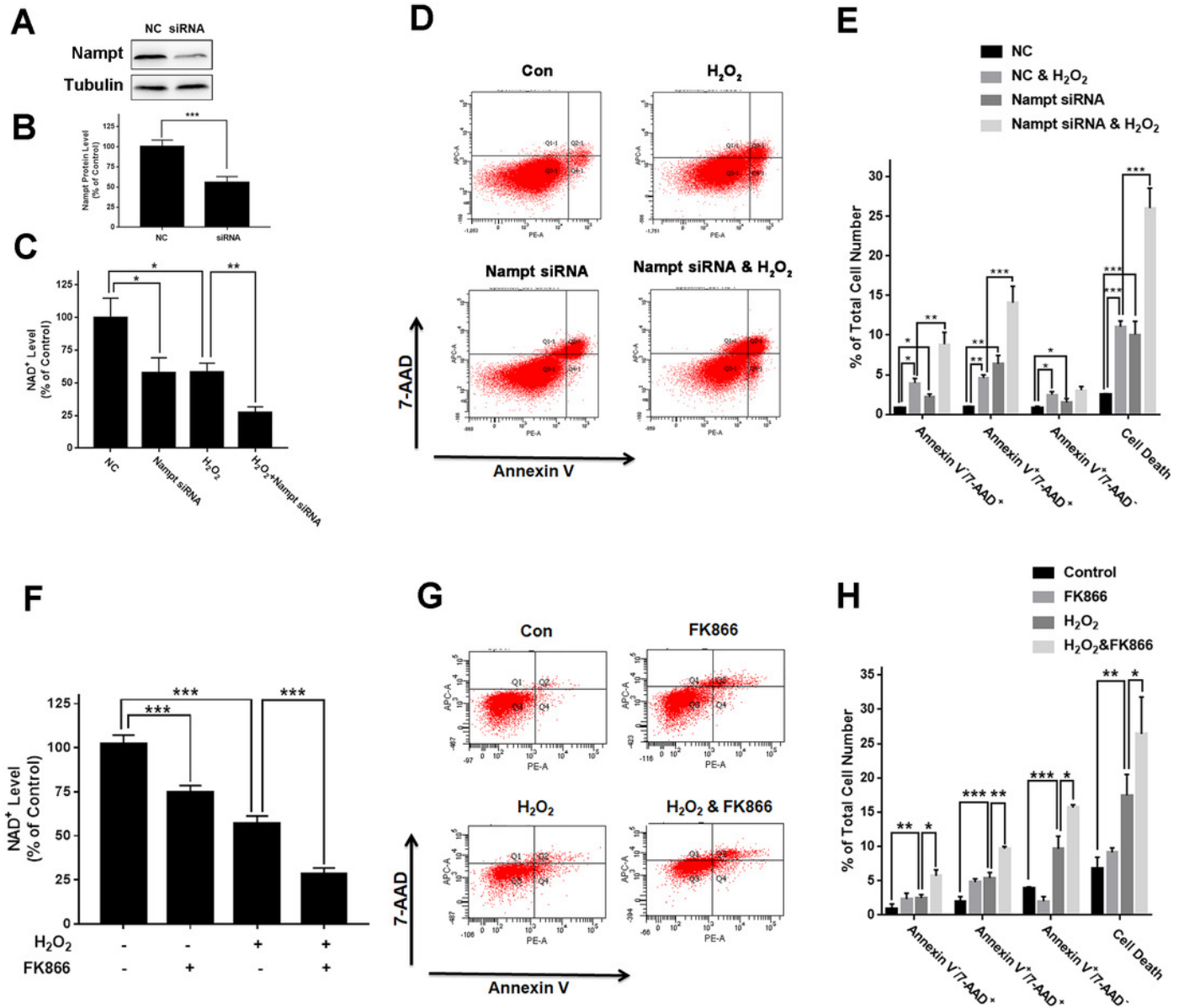
Figure 4

Fig. 4. Nampt activator P7C3 significantly attenuated H_2O_2-produced decreases in the intracellular NAD^+ levels and cell survival of differentiated PC12 cells.

(A) P7C3 significantly attenuated the H_2O_2-produced decrease in the intracellular NAD^+ levels of PC12 cells. (B, C) Nampt activator P7C3 significantly attenuated the H_2O_2-produced early-stage apoptosis (Annexin V^+/7-ADD$^-$) of the cells. PC12 cells were pre-treated with 10 mM P7C3 for 4 hrs, and then co-treated with 0.3 mM H_2O_2 for 6 hrs (for NAD^+ assay) or 20 hrs (for flow cytometry assay). The data were pooled from three independent experiments. $N = 3$. There were 3, 3 and 3 samples in these three independent experiments. $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.

A

NAD$^+$ Level (% of Control)

H$_2$O$_2$	−	−	+	+
P7C3	−	+	−	+

*** **

B

Con   P7C3

H$_2$O$_2$ P7C3 & H$_2$O$_2$

7-AAD

Annexin V

C

% of Total Cell Number

- Control
- P7C3
- H$_2$O$_2$
- P7C3 & H$_2$O$_2$

Annexin V$^-$/7-AAD$^+$ Annexin V$^+$/7-AAD$^+$ Annexin V$^+$/7-AAD$^-$ Cell Death

* *** ***   *** ***