

# p53-independent induction of p21<sup>Waf1/Cip1</sup> contributes to the activation of caspases in GTP-depletion-induced apoptosis of insulin-secreting cells

JX Huo<sup>1</sup>, SA Metz<sup>2</sup> and GD Li<sup>\*1</sup>

<sup>1</sup> Cardiovascular Research Institute, National University Medical Institutes, National University of Singapore, Singapore

<sup>2</sup> Pacific Northwest Research Institute, Seattle, WA 98122, USA

\* Corresponding author: GD Li, National University Medical Institutes, MD11 #02-01, 10 Medical Drive, Singapore 117597, Singapore.

Tel: +65-6874-5188; Fax: +65-6773-5461; E-mail: nmiligd@nus.edu.sg

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## Abstract

We investigated the role of some key regulators of cell cycle in the activation of caspases during apoptosis of insulin-secreting cells after sustained depletion of GTP by a specific inosine 5'-monophosphate dehydrogenase inhibitor, mycophenolic acid (MPA). p21<sup>Waf1/Cip1</sup> was significantly increased following MPA treatment, an event closely correlated with the time course of caspase activation under the same conditions. MPA-induced p21<sup>Waf1/Cip1</sup> was not mediated by p53, since p53 mass was gradually reduced over time of MPA treatment. The increment of p21<sup>Waf1/Cip1</sup> by MPA was further enhanced in the presence of a pan-caspase inhibitor, indicating that the increased p21<sup>Waf1/Cip1</sup> may occur prior to caspase activation. This notion of association of p21<sup>Waf1/Cip1</sup> accumulation with caspase activation and apoptosis was substantiated by using mimosine, a selective p21<sup>Waf1/Cip1</sup> inducer independent of p53. Mimosine, like MPA, also increased p21<sup>Waf1/Cip1</sup>, promoted apoptosis and simultaneously increased the activity of caspases. Furthermore, knocking down of p21<sup>Waf1/Cip1</sup> transfection of siRNA duplex inhibited caspase activation and apoptosis due to GTP depletion. In contrast to p21<sup>Waf1/Cip1</sup>, a reduction in p27<sup>Kip1</sup> occurred in MPA-treated cells. These results indicate that p21<sup>Waf1/Cip1</sup> may act as an upstream signal to block mitogenesis and activate caspases which in turn contribute to induction of apoptosis.

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**Abbreviations:** CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; GN, guanine nucleotide; IMPDH, inosine 5'-monophosphate dehydrogenase; MPA, mycophenolic acid; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride

## Introduction

Guanine nucleotides (GNs), and in particular GTP, modulate many biochemical reactions and their concentrations in the cell must be maintained at a critical level.<sup>1</sup> They are not only essential for the construction of DNA and RNA, but also important for the regulation of many cellular signaling pathways such as G-proteins and gene expression.<sup>2–4</sup> Inosine 5'-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) is the rate-limiting enzyme for GN biosynthesis, catalyzing the conversion of IMP into GMP. Mycophenolic acid (MPA) is a widely used and highly specific IMPDH inhibitor.<sup>5</sup> MPA depletes cellular GNs, which causes partial reduction in RNA synthesis and drastic inhibition of DNA synthesis, resulting in growth arrest and induction of apoptosis.<sup>2,5–8</sup> This effect can be reversed entirely by provision of guanine or guanosine but not of adenine or adenosine.<sup>7–9</sup>

It has been reported that inhibition of *de novo* GN synthesis by MPA and other IMPDH inhibitors slows cell proliferation by affecting the S phase of the cell cycle,<sup>8,10</sup> and suppresses the transition of cells from G0 to S phase in early- to mid-G1.<sup>9,11,12</sup> Cell cycle is coordinated by three families of molecules: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs).<sup>13</sup> CDKs are a group of protein kinases whose products must assemble into a holoenzyme with a cyclin subunit to become catalytically active. The function of cyclin-CDK complexes is regulated by phosphorylation of CDKs at different positions and by proteolysis of cyclins.<sup>13</sup> In mammalian cells, CDK2, 3, 4 and 6 play a role in the G1 and/or S phase while the CDK1 (cdc2) appears to be restricted to mitosis.<sup>14</sup> The expression and role of different cyclins are also distinct in the various phases of cell cycle. Cyclin B is critical for mitosis, while cyclin A is essential during the S phase and cyclins D and E are necessary in regulating the progression through G1.<sup>13,15</sup> To antagonize the actions of CDK-cyclin complexes in cell cycle, mammalian cells also express CDK inhibitors (CKIs) such as p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>, which bind to the complexes and lead to the prevention of their activation and inhibition of previously activated complexes.<sup>13,16</sup> Expression of these CKIs is induced by inhibitory regulators of cell cycle, and occurs during the normal state of growth arrest.<sup>16</sup>

Apoptosis may occur at any stages of cell cycle. However, the transition from G1 to S phase is regarded as a crucial point for deciding between cell growth and apoptosis. In particular,

cell progression from late G1 into S phase is regulated in many cells by p53 and by activation of CDKs.<sup>13,17,18</sup> The growth suppressor p53 is a tightly regulated transcription factor that can induce either cell cycle arrest or apoptosis dictated by its expression levels.<sup>19,20</sup> In addition, p53 is particularly important for protecting cells when DNA is damaged by radiation, chemicals or viral infection.<sup>21</sup> In this context, p53 is involved in the control of cell cycle in G1 and G2 phases through activation of different target genes such as those for p21<sup>Waf1/Cip1</sup>, GADD45 and 14-3-3.<sup>17,22,23</sup>

Two CDK inhibitors, p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>, are widely studied and are important for the regulation of cell cycle under various circumstances. It has been reported that p21<sup>Waf1/Cip1</sup> can act as either a downstream target/effector of p53 or independent of p53 to coordinate the cellular responses to negative growth signals.<sup>17,20,24</sup> p21<sup>Waf1/Cip1</sup> is a universal inhibitor of all known cyclin-CDK complexes.<sup>25</sup> An increase of p21<sup>Waf1/Cip1</sup> induces cell arrest at G1 and blocks cell entry into the S phase by inactivating CDKs or/and by inhibiting the activity of proliferating cell nuclear antigen (PCNA). p27<sup>Kip1</sup> shares homology at the N-terminal CDK-inhibitory domain with p21<sup>Waf1/Cip1</sup>.<sup>26,27</sup> This CKI interacts with the cyclin/CDK complexes and inhibits the kinase activity of cyclin A-CDK2, cyclin D-CDK4 and cyclin E-CDK2.<sup>26,27</sup> p27<sup>Kip1</sup> also regulates the progression through G1 and obstructs G1/S transition; this effect on blockage of cell cycle is, however, independent of p53.<sup>28</sup>

Several studies have suggested the importance of cell cycle molecules in islet  $\beta$ -cell growth and death, and possibly in the induction of diabetes.<sup>29-32</sup> We have previously demonstrated that sustained GN depletion, achieved by inhibiting IMPDH, interferes with DNA synthesis and induces a typical apoptosis of insulin-secreting cells that was characterized by morphological alternations, chromosome condensation and DNA fragmentation, as assessed by electron microscopy and DNA (oligonucleosomes) laddering.<sup>7</sup> Furthermore, we have recently reported that MPA arrests HIT-T15 cells at the G1 phase and triggers caspase-dependent apoptosis in which caspase-2 plays a major role.<sup>33</sup>

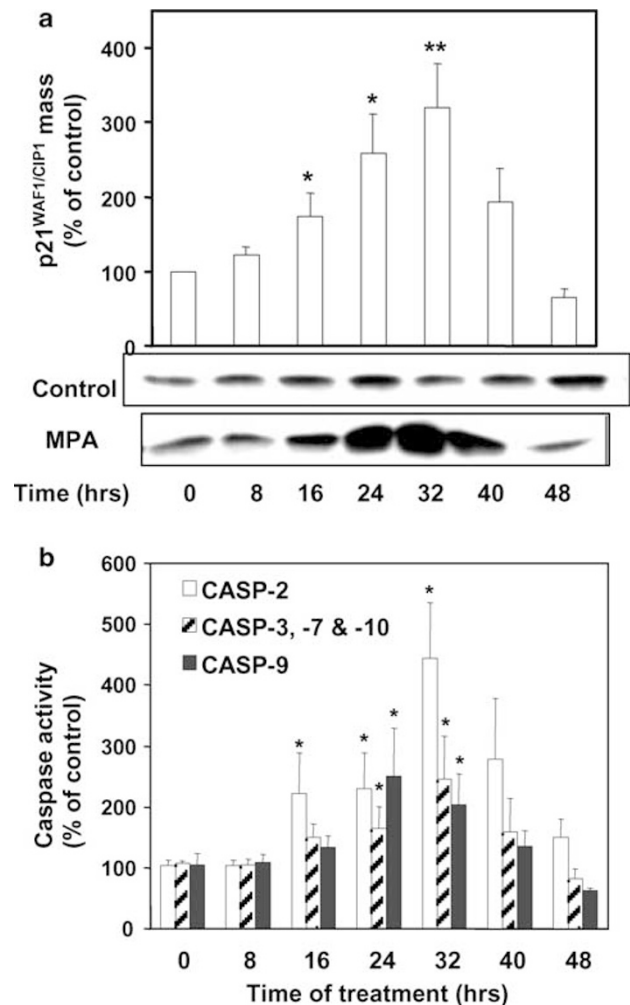
Caspases belong to a family of aspartate-specific cysteine proteases which play a central role in the execution of apoptotic death.<sup>34</sup> However, little is known about how GN depletion causes inhibition of mitogenesis and activation of caspases in particular. Interestingly, p53 may interact with IMPDH since the former downregulates IMPD activity, protein and mRNA expression of the latter.<sup>6,35,36</sup> It is possible (but previously unexplored) that the expression of some cell-cycle-regulating proteins may be changed and implicated in  $\beta$ -cell apoptosis triggered by GTP depletion. In the current study, we investigated the possible role of three cell cycle regulators (p53, p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>) in MPA-induced apoptosis of insulin-secreting cells, in particular their relationship with the activation of caspases. We found that p21<sup>Waf1/Cip1</sup> was increased in a close relationship with GTP-depletion-induced caspase activation and apoptosis, apparently without the involvement of p53 and p27<sup>Kip1</sup>. Also, we demonstrated for the first time that a selective p21<sup>Waf1/Cip1</sup> inducer, mimosine, can cause apoptotic cell death mediated by caspase activation. The fact that selective knockdown of p21<sup>Waf1/Cip1</sup> by siRNA transfection inhibited GTP depletion-induced caspase activa-

tion and apoptosis provided further evidence for the implication of p21<sup>Waf1/Cip1</sup> in this scenario. These data strongly indicated the important role of p21<sup>Waf1/Cip1</sup> in the induction of apoptosis of insulin-secreting cells due to sustained GN depletion and to other hostile challenges such as oxidative stress.<sup>29,31</sup>

## Results

### Increment of p21<sup>Waf1/Cip1</sup> by MPA treatment

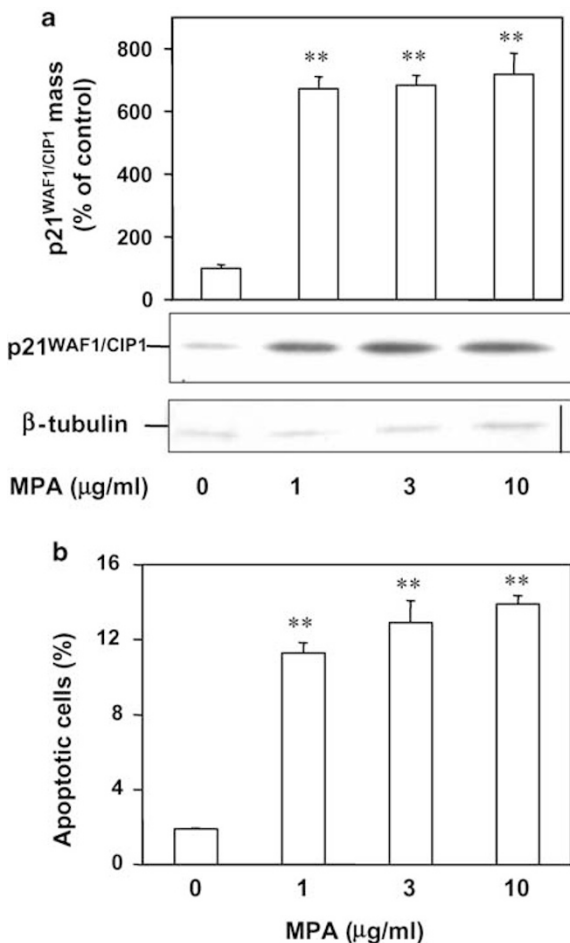
GTP depletion by MPA treatment significantly increased p21<sup>Waf1/Cip1</sup> (Figure 1a); its mass was elevated by increments



**Figure 1** Close correlation of time-response between the increment of p21<sup>Waf1/Cip1</sup> mass (a) and activation of caspases (b) in MPA-treated HIT-T15 cells. (a) Cells in culture were treated by 3  $\mu$ g/ml MPA for 0–48 h. At the indicated time points, cell lysates were prepared as described in 'Materials and methods'. Equal amounts of lysates (20  $\mu$ g protein extracts) were subjected to SDS-PAGE and blotted with an antibody against p21<sup>Waf1/Cip1</sup>. The mass of p21<sup>Waf1/Cip1</sup> was quantified by computer-assisted densitometry. (b) Cells seeded in six-well plates were treated with 3  $\mu$ g/ml MPA in culture media for 8–48 h. Caspase activity in cell homogenates was determined by measurement of the cleavage of the fluorogenic, specific substrates, as described in detail in the Materials and methods section. The values are means  $\pm$  S.E.M. of eight (a) and at least four (b) independent experiments. \* $P$ <0.05 and \*\* $P$ <0.01 versus zero time point (a) and versus control (0  $\mu$ g/ml MPA) (b)

of 23, 74, 159, 220 and 94% following 8, 16, 24, 32 and 40 h of MPA (3  $\mu$ g/ml) treatment, respectively. Furthermore, the pattern of time course of p21<sup>Waf1/Cip1</sup> induction was closely correlated with that of caspase activation (Figure 1b) occurring under the same conditions. Both events were significantly apparent at 16 h and reached maximal effects at 32 h of MPA exposure. These findings suggest that an increase in p21<sup>Waf1/Cip1</sup> expression may be involved in caspase activation.

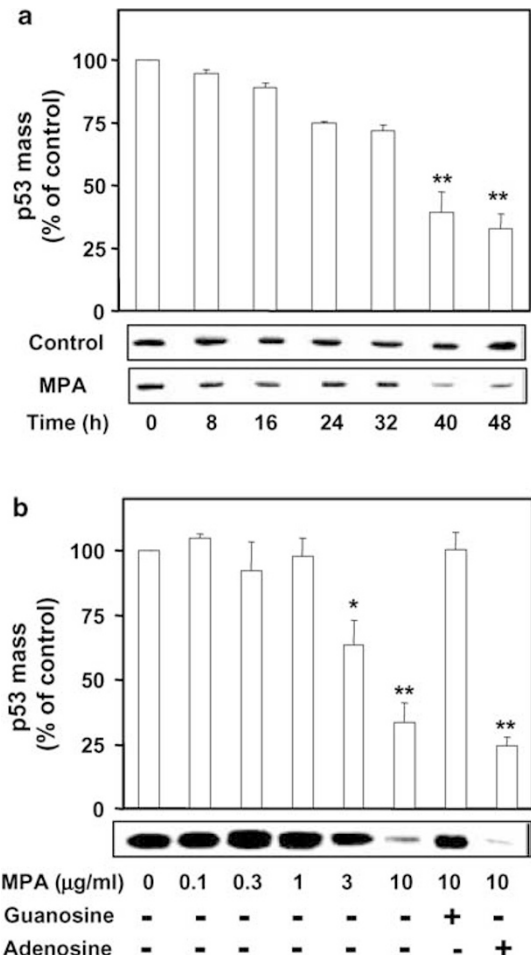
Depletion of GTP by MPA in another  $\beta$ -cell line, RINm5F, also induced a significant increase of p21<sup>Waf1/Cip1</sup> and caused apoptosis (Figure 2). RINm5F cells were even more susceptible to MPA treatment than HIT cells, since the effects reached were nearly maximum at 1  $\mu$ g/ml of MPA in these cells. In addition, treatment of RINm5F cells with 3  $\mu$ g/ml of MPA for 24 h increased caspase-2 activity by 109% ( $4.8 \pm 0.28$  in MPA-treated *versus*  $2.3 \pm 0.11$  arbitrary unit in control cells;  $n=3$ ;  $P<0.01$ ).



**Figure 2** Increment of p21<sup>Waf1/Cip1</sup> mass (a) and promotion of apoptosis (b) in MPA-treated RINm5F cells. Cells in culture were treated with 0–10  $\mu$ g/ml MPA for 24 h. (a) Equal amounts of cell lysates (20  $\mu$ g protein extracts) were subjected to SDS-PAGE, and blotted with an antibody against p21<sup>Waf1/Cip1</sup>. The mass of p21<sup>Waf1/Cip1</sup> was quantified by computer-assisted densitometry. (b) Apoptosis of cells was assessed by PI staining, followed by flow cytometric analysis. The cells whose DNA content was lower than the G1 phase (sub-G1) contained fragmented nuclei, and were undergoing subdiploidy apoptosis. The values are means  $\pm$  S.E.M. of six (a) and three (b) independent experiments. \*\* $P<0.01$  *versus* control (0  $\mu$ g/ml MPA)

### Reduction of p53 and p27<sup>Kip1</sup> during MPA treatment

Transcription of the p21<sup>Waf1/Cip1</sup> gene is activated by both p53-dependent and -independent mechanisms.<sup>17,24,37–39</sup> Thus, we next examined the p53 profile under the conditions when MPA caused an increase of p21<sup>Waf1/Cip1</sup>. Our results revealed that the levels of p53 protein in HIT cells were progressively decreased with the treatment time of MPA (10  $\mu$ g/ml): reduction by 5, 11, 25, 28, 34 and 58% after exposure to MPA for 8, 16, 24, 32, 40 and 48 h, respectively (Figure 3a). In addition, this effect occurred in a dose-dependent manner; p53 mass was significantly decrease by 37 and 64% at 3 and 10  $\mu$ g/ml of MPA treatment for 48 h, respectively (Figure 3b). Coculture with guanosine (500  $\mu$ M), but not by adenosine (500  $\mu$ M), entirely reversed the p53 reduction due to GN depletion by MPA. These observations indicate that the



**Figure 3** Reduction of p53 protein by MPA treatment. HIT-T15 cells were cultured in RPMI medium containing 10  $\mu$ g/ml MPA for 0–48 h (a) or different concentrations of MPA for 48 h (b). When present, 0.5 mM guanosine or adenosine was also included. Cell lysates were prepared and subject to SDS-PAGE (20  $\mu$ g protein each) for separation. Western blotting was performed by blotting the membranes with the sheep polyclonal, peroxidase-conjugated antibody specific for wild p53. The blots were analyzed by computer-assisted densitometry. Values are means  $\pm$  S.E.M. of five (a) and four (b) independent experiments. \* $P<0.05$ , \*\* $P<0.01$  *versus* zero-hour point (in a) and *versus* control (0  $\mu$ g/ml MPA) (b)

increase of p21<sup>Waf1/Cip1</sup> by GN-depletion due to MPA treatment in HIT cells is achieved via a mechanism independent of p53.

p27<sup>Kip1</sup> is another important CKI regulating the progression through G1 and the G1/S transition.<sup>26,27</sup> In contrast to the increase of p21<sup>Waf1/Cip1</sup>, MPA treatment caused progressive decreases of the mass of p27<sup>Kip1</sup> (original data not shown). After treatment for 16, 24, 32, 40 and 48 h with 3  $\mu$ g/ml MPA, p27<sup>Kip1</sup> levels declined by 34, 55, 72, 74 and 75%, respectively. This declining effect was also dose-dependent, with significant reductions of 41, 61 and 68% following 48-h treatment at 1, 3 and 10  $\mu$ g/ml MPA, respectively. Thus, there is a drop in both p53 and p27<sup>Kip1</sup> after GN depletion, while the latter is a bit more sensitive (occurring at earlier treatment time (32 h) and at lower concentrations of MPA (1  $\mu$ g/ml)).

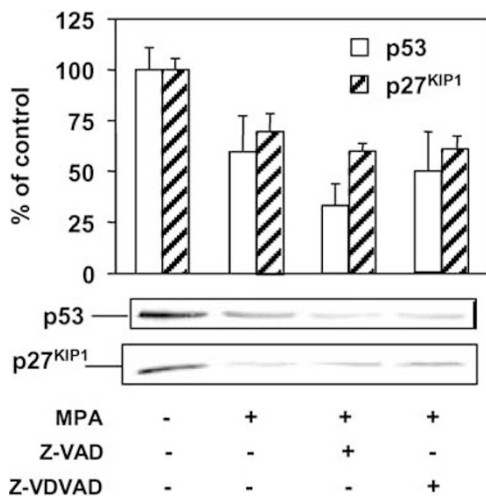
Interestingly, these MPA effects on p53 and p27<sup>Kip1</sup> could not be reversed by cotreatment with either a general caspase inhibitor (Z-VAD-FMK) or a specific caspase-2 inhibitor (Z-VDVAD-FMK) (Figure 4), although both caspase inhibitors are able to block the apoptosis triggered by MPA treatment.<sup>33</sup> These results indicate that GN depletion by MPA causes decrease of p53 and p27<sup>Kip1</sup> independent of caspase(s) activation.

### p21<sup>Waf1/Cip1</sup> and caspase activation

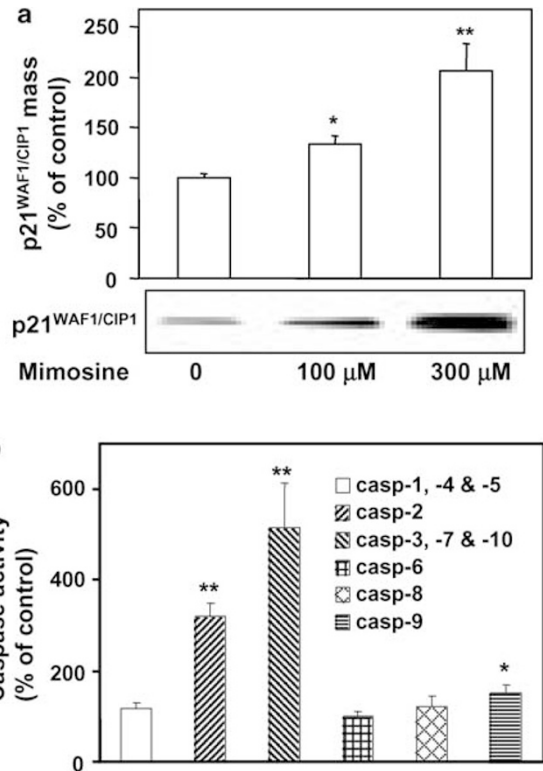
The results described above demonstrated that GTP depletion by MPA induced p21<sup>Waf1/Cip1</sup> increase in close correlation with caspase activation in a p53-independent mechanism. It is known that p21<sup>Waf1/Cip1</sup> can serve as a critical checkpoint regulator for both cell cycle arrest and apoptosis.<sup>40,41</sup> Thus, we postulated that p21<sup>Waf1/Cip1</sup> might mediate the caspase activation occurring during GTP-depletion-induced apoptosis. To test this hypothesis, we utilized a specific inducer of p21<sup>Waf1/Cip1</sup>, mimosine, to mimic the action of MPA. Our choice of mimosine was based on its ability to increase both

p21<sup>Waf1/Cip1</sup> mRNA and protein levels independent of p53,<sup>38,42</sup> an effect similar to that of MPA. It has been reported that this agent potentially causes a reversible block of the cell cycle at late G1 phase and is frequently used to achieve cell synchronization at this stage.<sup>38,43,44</sup> It was also found that mimosine can interfere with deoxyribonucleotide metabolism and inhibit DNA synthesis.<sup>45</sup>

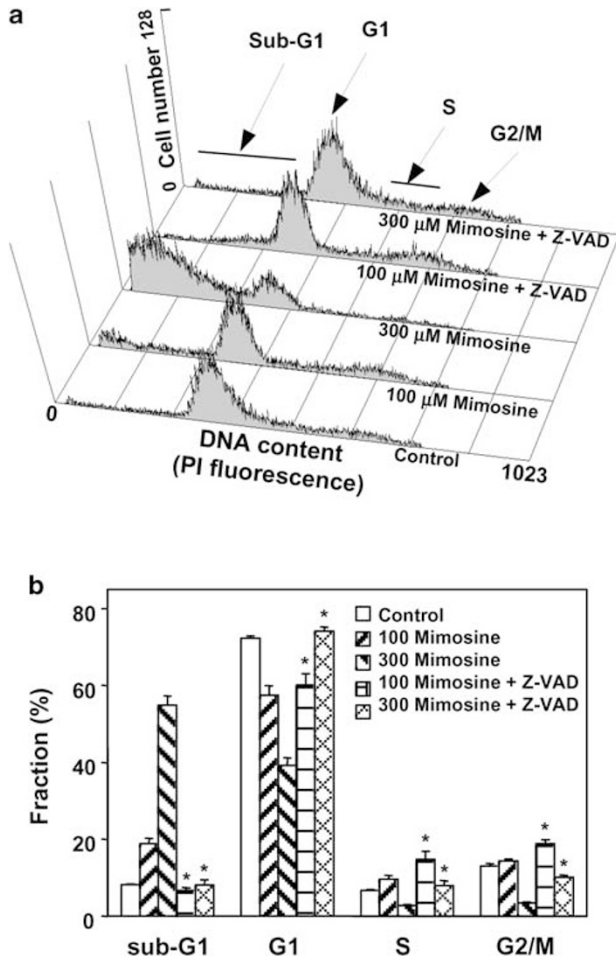
We found that treatment of mimosine for 32 h significantly increased p21<sup>Waf1/Cip1</sup> by 35 and 116% at 100 and 300  $\mu$ M, respectively (Figure 5a). Importantly, mimosine (300  $\mu$ M) also simultaneously increased activity of caspase-2, -3 and -9 by 220, 416 and 52%, respectively (Figure 5b), thereby mimicking MPA effects. Furthermore, flow cytometric analysis revealed that mimosine inhibited the progression of cells from G1 to G2/M phase and promoted apoptosis, as reflected by DNA fragmentation (an increase in sub-G1 fraction) (Figure 6a). The number of apoptotic cells (sub-G1) was significantly increased by 1.3- and 5.6-fold ( $P < 0.01$ ) after treatment for 32 h with 100 and 300  $\mu$ M mimosine, respectively (Figure 6b). In addition, cells in S and G2/M phases were significantly reduced by 57 and 74% at 300  $\mu$ M mimosine (Figure 6b). These cell cycle results are similar to our previous findings with MPA treatment.<sup>33</sup> Importantly, cotreatment of cells with the pan-caspase inhibitor Z-VAD-FMK (100  $\mu$ M) could completely block mimosine-induced apoptosis and



**Figure 4** No restorative effect by caspase inhibitors on MPA-induced reduction of p53 and p27<sup>Kip1</sup>. HIT cells were treated with 10  $\mu$ g/ml (for p53) or 3  $\mu$ g/ml (for p27<sup>Kip1</sup>) MPA in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) or a caspase-2 inhibitor (Z-VDVAD-FMK) in culture for 32 h. See the Materials and Methods section for other experimental details. Values are means  $\pm$  S.E.M. of four (for p53) and 10 (for p27<sup>Kip1</sup>) independent experiments



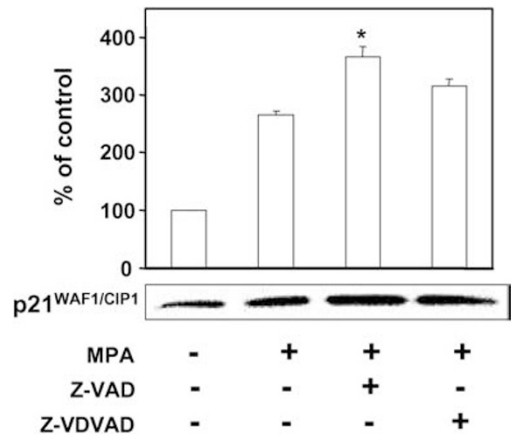
**Figure 5** Induction of p21<sup>Waf1/Cip1</sup> (a) and activation of caspases (b) by mimosine treatment. HIT cells were treated with mimosine in culture for 32 h. The levels of p21<sup>Waf1/Cip1</sup> in cell lysates (20  $\mu$ g protein extracts each) were determined by Western blotting and analyzed by computer-assisted densitometry. Caspase activity in cell homogenates was assessed by measuring the cleavage of the fluorogenic, specific substrates, as described in detail in 'Materials and methods'. Values are means  $\pm$  S.E.M. of four (a) and three (b) independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  versus control



**Figure 6** Blockage by a caspase inhibitor of mimosine-induced arrest of cell cycle and induction of apoptotic cell death. HIT cells seeded on six-well plates were treated with mimosine in culture media for 32 h. Cells were stained with PI and subjected to flow cytometric analysis of mimosine-induced subdiploidy apoptotic cells. The cells whose DNA contents were lower than the G1 phase (sub-G1) contained fragmented nuclei and were undergoing apoptosis. The graph is representative of four independent experiments. The data in (b) show the statistical analysis of the mimosine effects on apoptosis and cell cycle. The results are expressed as percentages of total cells (10 000) evaluated by flow cytometry. \* $P < 0.01$  versus individual mimosine treatment alone

restore the arrest in cell cycle (Figure 6a and b). However, mimosine-induced cell arrest and apoptosis was not dependent upon GTP depletion, since cotreatment of cells with guanosine (500  $\mu$ M) could not prevent the effects of mimosine (data not shown). These data strongly indicate that p21<sup>Waf1/Cip1</sup> may be the mediator acting as an upstream signal to activate caspases and in turn induce apoptosis during MPA treatment or other conditions of programmed cell death.

It has been reported that p21<sup>Waf1/Cip1</sup> is a substrate for executive caspases such as caspase-3, and this kind of degradation can be reversed by specific caspase inhibitors.<sup>40,46</sup> A similar phenomenon was also observed in our study. We found that the general caspase inhibitor Z-VAD-FMK, but not the caspase-2 inhibitor Z-VDVAD-FMK, enhanced the increment (+38%) of p21<sup>Waf1/Cip1</sup> due to MPA treatment (Figure 7). This observation again suggests that p21<sup>Waf1/Cip1</sup> induction by GTP depletion is an event



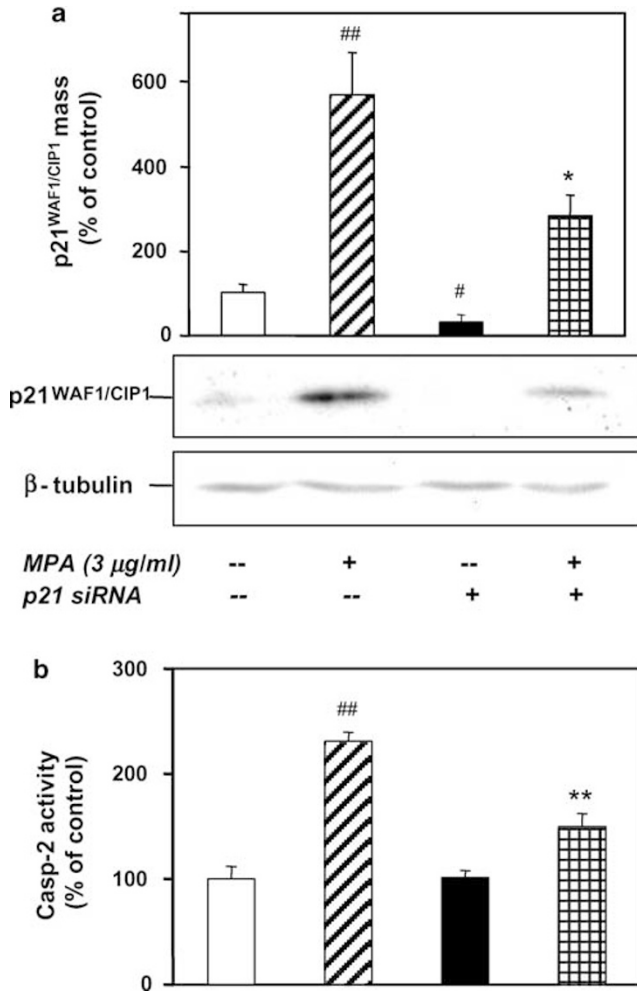
**Figure 7** Enhancement of MPA-induced p21<sup>Waf1/Cip1</sup> increment by caspase inhibitors. HIT cells were treated with MPA (3  $\mu$ g/ml) in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) or a caspase-2 inhibitor (Z-VDVAD-FMK) in culture for 32 h. p21<sup>Waf1/Cip1</sup> mass in cell lysates (20  $\mu$ g protein extracts each) was determined and analyzed as described in the legend to Figure 1. Values are means  $\pm$  S.E.M. of 13 independent experiments. \* $P < 0.05$  versus MPA treatment alone

upstream of caspase activation during apoptosis, although activated caspases may exert a constraining effect on p21<sup>Waf1/Cip1</sup> levels.

### Inhibition of MPA-induced caspase-2 activation and apoptosis by knockdown of p21<sup>Waf1/Cip1</sup> by siRNA transfection

In order to directly address the role of p21<sup>Waf1/Cip1</sup> in MPA-induced cell death, we employed the siRNA technique to selectively knock down p21<sup>Waf1/Cip1</sup>. By transfection of appropriate 21-nucleotide siRNA duplexes into cells, the RNA oligonucleotides will specifically suppress the expression of endogenous and heterologous genes.<sup>47</sup> However, the success of this approach requires the complete complementary match of siRNA with its targeted mRNA, since even one nucleotide mismatch would cause the failure of knockdown.<sup>47</sup> For this purpose, we have successfully decoded the 500 nucleotide sequences of the 5'-coding end of p21<sup>Waf1/Cip1</sup> cDNA in HIT cells (derived from hamster; the gene for p21<sup>Waf1/Cip1</sup> in this species is unavailable) by RT-PCR, using primers of rat p21<sup>Waf1/Cip1</sup> mRNA as probes. As indicated in the Materials and methods section, the decoded portion of cDNA for p21<sup>Waf1/Cip1</sup> in hamster is highly compatible with that in rat, mice and human (79–88% identity).

When HIT cells were transfected with a 21-mer siRNA duplex corresponding to 137–157 (from the start codon) of hamster p21<sup>Waf1/Cip1</sup> mRNA for 8 h and then treated with 3  $\mu$ g/ml of MPA for 32 h after 18-h recovery, both the basal level and MPA-induced induction of p21<sup>Waf1/Cip1</sup> were significantly knocked down by 68 and 50%, as demonstrated by Western blotting (Figure 8a). Importantly, the increment of caspase-2 activity due to GTP depletion was also significantly attenuated by 61%, in a fashion quantitatively similar to siRNA on p21 effects (Figure 8b). Moreover, GTP depletion-induced apoptotic death was inhibited in parallel (by 45%) in these cells (Table 1). The suppression of cell cycle due to GTP depletion



**Figure 8** Attenuation of the effects of GTP depletion on p21<sup>Waf1/Cip1</sup> induction, caspase-2 activation and apoptosis of HIT cells by transfection of p21<sup>Waf1/Cip1</sup> siRNA. HIT cells were transfected with control or p21<sup>Waf1/Cip1</sup> siRNA duplex for 8 h plus 18-h recovery and followed by treatment with MPA (3  $\mu$ g/ml) for 32 h. p21<sup>Waf1/Cip1</sup> mass (a) and caspase-2 activity (b) in cell lysates were determined and analyzed as described in the legend to Figure 1. Values are means  $\pm$  S.E.M. of at least three independent experiments. <sup>#</sup> $P < 0.05$  and <sup>##</sup> $P < 0.01$  versus control cells (0  $\mu$ g/ml MPA). <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  versus MPA treatment alone

**Table 1** Inhibition of MPA-induced apoptosis by transfection of p21<sup>Waf1/Cip1</sup> siRNA

	Fraction (%)		
	Sub-G1	G	S/G2/M
Control	10.6 $\pm$ 0.6	66.4 $\pm$ 1.0	23.7 $\pm$ 0.6
MPA (3 $\mu$ g/ml)	24.9 $\pm$ 0.6 <sup>a</sup>	57.9 $\pm$ 0.3 <sup>a</sup>	17.7 $\pm$ 0.6 <sup>a</sup>
p21-siRNA	8.6 $\pm$ 0.6	68.7 $\pm$ 0.5	23.0 $\pm$ 0.6
p21-siRNA+MPA	16.4 $\pm$ 0.6 <sup>b</sup>	63.6 $\pm$ 0.8 <sup>b</sup>	20.3 $\pm$ 0.7 <sup>b</sup>

HIT cells were transfected with control or p21<sup>Waf1/Cip1</sup> siRNA duplex for 8 h plus 18-h recovery and followed by treatment with MPA (3  $\mu$ g/ml) for 32 h. Apoptosis and cell cycle were assessed by flow cytometry as described in the legend to Figure 6. Values are means  $\pm$  S.E.M. of three independent experiments. <sup>a</sup> $P < 0.01$  versus control. <sup>b</sup> $P < 0.01$  versus MPA treatment alone

was also reduced by knockdown of p21<sup>Waf1/Cip1</sup> with siRNA transfection (Table 1). These data provide direct evidence for the implication of p21<sup>Waf1/Cip1</sup> in GTP depletion-induced caspase-2 activation and apoptosis in  $\beta$  cells.

## Discussion

Several studies have demonstrated that the inhibition of IMPDH potently inhibits DNA synthesis and arrests cell cycle, possibly followed by apoptosis or induction of differentiation of cultured cells.<sup>2,5,6,8</sup> We have also observed that specific GTP depletion by MPA restrained the mitogenesis of insulin-secreting cells by reducing their progression from G1 phase into S and G2/M phases, resulting in apoptosis mediated by activation of caspases.<sup>7,33</sup> In the present study, we defined the linkage between cell cycle arrest and the induction of apoptosis induced by MPA treatment in insulin-secreting cells. We found that MPA induced p21<sup>Waf1/Cip1</sup> expression, which was closely correlated with caspase activation and apoptosis in two widely used  $\beta$ -cell lines. In addition, a specific p21<sup>Waf1/Cip1</sup> inducer, mimosine, could mimic the MPA effects on activation of caspases, resulting in apoptosis (but in a GTP-independent manner). Furthermore, direct evidence for the involvement of p21<sup>Waf1/Cip1</sup> in the mediation of this scenario was obtained via the knockdown of p21<sup>Waf1/Cip1</sup> using siRNA transfection, since caspase activation and apoptosis induced by GTP depletion were inhibited by this maneuver. To our knowledge, this is the first study systematically investigating p21<sup>Waf1/Cip1</sup> and establishing its relationship to caspase activation during apoptosis induced by GTP depletion.

It is well known that CDIs are able to inhibit cell proliferation by negatively affecting cyclin-CDK complexes. By doing so, p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> regulate the progression through G1 and the G1/S transition.<sup>26,27</sup> Since depletion of cellular GNs by MPA inhibits cell proliferation and arrests the cell cycle in G1 phase,<sup>9,11,33</sup> it is possible that MPA may alter p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> expression to achieve this effect. Only one study has mentioned the induction of p21<sup>Waf1/Cip1</sup> by MPA (1  $\mu$ M) in T lymphocytes and this effect occurred at 42 h, but not 24-h, treatment with MPA.<sup>11</sup> In the present study, we found that depletion of GNs by MPA treatment caused increases of p21<sup>Waf1/Cip1</sup> following a time course which was in parallel with the activation of several caspases in HIT cells (cf. Figure 1), suggesting a close relationship between the two events. Importantly, significant accumulation of p21<sup>Waf1/Cip1</sup> and activation of caspases was observed as early as 16 h of MPA treatment and thus preceding the occurrence of apparent apoptosis (at 24 h) of HIT cells.<sup>7,33</sup>

To further examine the possible sequence of observed caspase activation and p21<sup>Waf1/Cip1</sup> accumulation, we used mimosine, a well-known p21<sup>Waf1/Cip1</sup> inducer, to study its effects on the two events. By increasing p21<sup>Waf1/Cip1</sup>, this compound has been found to be a potent, reversible inhibitor which synchronizes cells in the late G1 phase.<sup>38,43,44</sup> Mimosine increased both p21<sup>Waf1/Cip1</sup> mRNA and protein levels, bypassing the requirement for transcriptional activation by p53.<sup>38,42</sup> However, there is no study reporting its effect on caspases and apoptosis directly. In the current study, we found that mimosine had effects similar to those of MPA in

increasing p21<sup>Waf1/Cip1</sup> and inducing activation of the same types of caspases.<sup>33</sup> Moreover, under these conditions, mimosine suppressed the cell cycle and promoted caspase-mediated apoptotic death of HIT cells, the same effects observed after MPA treatment.<sup>33</sup> Although it has been reported that mimosine inhibits deoxyribonucleotide metabolism,<sup>45</sup> we found that its effects on cell arrest and apoptosis are not due to a depletion of GTP.

Our experiments using caspase inhibition further uncovered a relationship between p21<sup>Waf1/Cip1</sup> and caspase activation. When caspases were suppressed by a pan-caspase inhibitor, p21<sup>Waf1/Cip1</sup> mass was even modestly enhanced during MPA treatment (Figure 7), indicating that GN depletion can induce p21<sup>Waf1/Cip1</sup> without caspase activation – in other words, prior to caspase activation. However, inhibition of caspases had no effects on p53 and p27<sup>Kip1</sup> levels. More direct evidence for the important role of p21<sup>Waf1/Cip1</sup> in GTP depletion-induced caspase activation and apoptosis come from the results of selective knockdown of p21<sup>Waf1/Cip1</sup> by siRNA transfection. Both the basal level and MPA-induced induction of p21<sup>Waf1/Cip1</sup> were dramatically reduced in the siRNA-transfected cells. Importantly, the increment of caspase-2 activity due to GTP depletion was also attenuated in parallel. Consequently, GTP depletion-induced apoptotic death was significantly inhibited in these cells. The failure of complete blockade of GTP-depletion effects (on caspase-2 activation and apoptosis) by siRNA transfection in cell populations is most likely due to the incomplete knockdown of p21<sup>Waf1/Cip1</sup> because of the incomplete transfection efficiency, although we cannot exclude the possibility that another factor is involved in this cascade of events in addition to p21. All these observations strongly suggest that p21<sup>Waf1/Cip1</sup> may act as an upstream signal to activate caspases. Therefore, we can infer that sustained GTP depletion significantly increased p21<sup>Waf1/Cip1</sup> accumulation, which triggered a cascade of activation of caspases leading to apoptosis, since GTP repletion reversed these effects.

The observation of enhanced p21<sup>Waf1/Cip1</sup> accumulation in the presence of a pan-caspase inhibitor also implies that p21<sup>Waf1/Cip1</sup> may be degraded by activated caspases. Indeed, there is evidence that p21<sup>Waf1/Cip1</sup> may be a substrate of effector caspases in the late stage of apoptosis.<sup>41,46</sup> The significance of this phenomenon, however, is unclear. It might be a negative feedback mechanism whereby the cell protects itself from damage by shutting off the upstream signals. The nonalteration of MPA-induced reduction of p27<sup>Kip1</sup> and p53 by the caspase inhibitor also suggests that these effects are the direct results of GTP depletion. These phenomena might not play a critical role in caspase activation and apoptosis due to GTP depletion, since both the molecules were reduced and thus not responsible for the inhibition of mitogenesis in our cells.

Ample evidence demonstrated that induction of p21<sup>Waf1/Cip1</sup> can be achieved by either the p53-dependent<sup>17,37</sup> or -independent pathway.<sup>38,39</sup> In lymphocytes, 24-h treatment with MPA (1  $\mu$ M) resulted in a very low level of p53 expression.<sup>11</sup> In our study, the p21<sup>Waf1/Cip1</sup> induction was apparently not mediated by p53, since the latter was not increased but rather progressively reduced by MPA treatment in both time- and dose-dependent manners. Thus, p53

may not be an important mediator for MPA-induced apoptosis.

IMPDH is a key enzyme for biosynthesis of GNs.<sup>2</sup> Its gene expression is regulated inversely by a post-transcriptional nuclear event in response to fluctuations in the intracellular level of GNs.<sup>48</sup> As a specific inhibitor of IMPDH, MPA diminished GNs levels and, therefore, increased the levels of IMPDH mRNA and amounts of the enzyme.<sup>4,48</sup> In addition, IMPDH activity and its protein and mRNA levels could be downregulated by p53 and, thus, this enzyme (or the resultant changes in cellular levels of GNs) might play an important role in mediating p53-dependent suppression of cell growth.<sup>3,6,35,36</sup> The reciprocal effect of IMPDH on p53 has not been reported. However, our findings that p53 was decreased by GTP depletion (which would increase IMPDH expression<sup>4</sup>) suggested such a possible consequence. This notion was further supported by the results from using caspase inhibitors. Under these conditions, caspase activation and apoptosis due to MPA were blocked while GNs remained depleted<sup>33</sup> and the effect of reducing p53 was not affected (cf. Figure 4a). Only restoration of GNs by guanosine was able to prevent p53 reduction (cf. Figure 3b). Thus, we can conclude that p53 decrement in HIT cell during MPA-induced apoptosis is attributable to GTP depletion. Alternatively, the reduction of p53 mass may be due to the degradation by some unknown proteases activated by GN depletion.

Little information on the effect of GN on p27<sup>Kip1</sup> is available. One study reported that MPA prevented the IL-2-induced elimination of p27<sup>Kip1</sup> and resulted in the retention of high levels of p27<sup>Kip1</sup> in IL-2/leucoagglutinin-treated T cells.<sup>11</sup> Our data revealed that MPA-induced arrest of HIT cells may not be mediated by p27<sup>Kip1</sup> since the mass of this CKI was actually reduced by MPA in a dose- and time-dependent manner. In addition, p27<sup>Kip1</sup> was not a substrate of caspases activated by GN depletion, since the reduction of p27<sup>Kip1</sup> was not reversed by caspase inhibitors, suggesting an effect possibly related to GN depletion directly. Whether this reduction was due to decreased expression or to increased degradation of the protein is unclear. The level of p27<sup>Kip1</sup> is regulated in several ways;<sup>49</sup> the precise biochemical link between GTP depletion and p27<sup>Kip1</sup> reduction in islet  $\beta$  cells remains to be clarified in further study. Interestingly, in this study, we found that both p53 and p27<sup>Kip1</sup> were decreased (though the latter was more sensitive) following MPA treatment; but it is not known whether any relationship exists between the two events based on the data from this study and the studies reported in the literature.

Cell cycle molecules play important roles in islet  $\beta$ -cell growth and death and the development of diabetes. It has been found that Cdk4 (which forms a complex with cyclin D1 and is required for the progression of cell cycle from G1 to S phase) is essential for islet  $\beta$ -cell growth and postnatal survival, and that the loss of its expression causes insulin-deficient diabetes in laboratory models.<sup>30,32</sup> Degeneration of pancreatic islets by apoptosis occurs in the Cdk4 knockout mice after birth,<sup>32</sup> whereas the mice expressing a Cdk4 mutant unable to be inhibited by a CKI exhibited hyperplasia of pancreatic islets due to enhanced  $\beta$ -cell proliferation.<sup>30</sup> In addition, elevated glucose concentrations impair proliferation

and induce the apoptosis of  $\beta$  cells. High glucose can generate multiple proapoptotic molecules in  $\beta$  cells, including oxidative stress,<sup>29,50,51</sup> promotion of rising cytosolic free  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ),<sup>52</sup> upregulation of Bax proteins and Fas receptor<sup>50,53</sup> and induction of inflammatory cytokines.<sup>54</sup> All these events can result in  $\beta$ -cell death by apoptosis probably through activation of caspases.<sup>50,55</sup> The possible role of p21<sup>Waf1/Cip1</sup> in  $\beta$ -cell apoptosis due to glucotoxicity is evidenced by the study that has clearly demonstrated that oxidative stress is able to induce p21<sup>Waf1/Cip1</sup> in  $\beta$  cells.<sup>29</sup> Oxidative stress also causes p53-independent activation of p21<sup>Waf1/Cip1</sup> expression in non- $\beta$  cells.<sup>56</sup> In another study, enhanced expression of p53 and p21<sup>Waf1/Cip1</sup> occurred in amylin-induced apoptosis of insulin-secreting (RINm5F) cells.<sup>31</sup>

In conclusion, our results demonstrated that reduction of GNs induces p53-independent p21<sup>Waf1/Cip1</sup> expression and that this induction of p21<sup>Waf1/Cip1</sup> may mediate the arrest of cell cycle, activation of caspases and, in turn, apoptotic cell death of insulin-secreting cells. Our findings in this study and the results by others<sup>29,31</sup> have indicated that p21<sup>Waf1/Cip1</sup> might play an important role in the induction of islet  $\beta$ -cell apoptosis.

## Materials and methods

### Materials

Most of the reagents and chemicals used in this study were purchased from the companies listed as follows: MPA, adenosine, guanosine, mimosine, CHAPS, HEPES, PMSF, EDTA, DTT, pepstatin A, aprotinin and RPMI 1640 were from Sigma, St. Louis, MO, USA; ammonium persulphate, bromophenol blue and TEMED were from Pharmacia Biotech AB, Sweden; fetal calf serum was from Gibco BRL, Life Technologies, Rockville, MD, USA; anti-p53-protein-POD, BSA and leupeptin were from Roche Molecular Biochemicals, Mannheim, Germany; Bio-Rad Protein Assay Dye Reagent, protein standard, PVDF membrane, caspase (1–10) substrates, general caspase inhibitor (Z-VAD-FMK) and caspase-2 inhibitor (Z-VDVAD-FMK) were supplied by Bio-Rad Laboratories, Hercules, CA, USA; affinity-purified polyclonal antibody (from goat) of p21<sup>Waf1/Cip1</sup>, affinity-purified polyclonal antibody (from goat) of p27<sup>Kip1</sup> and anti-goat IgG-HRP were from Santa Cruz Biotechnology, Santa Cruz, CA, USA and BD Biosciences, Franklin Lakes, NJ, USA.

### Cell culture

Insulin-secreting HIT-T15 cells (passages 76–83) and RINm5F cells were maintained in Falcon dishes in RPMI 1640 supplemented with 10% decompartmented fetal calf serum (v/v), 100 i.u. penicillin/ml and 100  $\mu\text{g}$  streptomycin/ml. Cells were seeded at a density of  $1.0 \times 10^6/\text{ml}$  in culture dishes or multiwell plates, and the medium was changed every 48 h. MPA and other test agents were added to cells when they reached more than 80% confluence.

### Induction of apoptosis by GTP depletion

MPA stock solution (2 mg/ml) was prepared in ethanol and added into cell culture medium to final concentrations of 0.1–10  $\mu\text{g}/\text{ml}$  up to 48 h to induce apoptosis as described previously.<sup>7,33</sup>

### Flow cytometric analysis for DNA fragmentation and cell cycle

DNA fragmentation and cell cycle were determined by propidium iodide (PI) staining and flow cytometry as described by Nicoletti *et al.*,<sup>57</sup> and in detail in our previous study.<sup>33</sup> This assay measures fragmented nuclei and thus detects the subdiploidy apoptosis.<sup>57</sup> Briefly, HIT or RINm5F cells were cultured in six-well plates with test agents for the indicated periods. All cells in the wells were collected and fixed in 70% ethanol. For PI staining, the ethanol-suspended cells were centrifuged for 5 min at  $200 \times g$  to remove cell debris. Afterward, the cell pellets were incubated in 1 ml PI/Triton X-100 staining solution (containing 20  $\mu\text{g}/\text{ml}$  PI, 0.1% Triton X-100 and 0.2 mg/ml RNase A in PBS) for 30 min at room temperature. Flow cytometric analysis was carried out using a fluorescence-activated – cell sorter (EPICS Elite ESP, Beckman Coulter, Hialeah, FL, USA). In all, 10 000 cells were evaluated for each sample. The data were processed and analyzed by using WinMDI software (Scripps Institute, La Jolla, CA, USA).

### Caspase activity measurement

Measurements of caspase activity are simplified by using fluorogenic synthetic oligopeptide substrates.<sup>33,58</sup> These oligopeptides are identical or similar to those found in full-length protein substrates, and, where differences occur, the optimized synthetic substrates appear to be cleaved as efficiently or better than the full-length proteins. The synthesized oligopeptide substrates are modified at the caspase cleavage site (C-terminal aspartic acid) with 7-amino-4-trifluoromethyl coumarin (AFC). When liberated from the peptide, AFC produces an optical change resulting in emissions of fluorescence.

HIT or RINm5F cells were cultured in six-well plates. For each independent experiment, duplicates of both control and treated wells were studied for any time point of treatment. After decanting the medium and rinsing with cold PBS, 100  $\mu\text{l}$  lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 350  $\mu\text{g}/\text{ml}$  PMSF, 10  $\mu\text{g}/\text{ml}$  pepstatin A, 10  $\mu\text{g}/\text{ml}$  aprotinin and 20  $\mu\text{g}/\text{ml}$  leupeptin) was added to each well. Cells were scraped with a cell lifter and transferred to clean tubes. Afterward, the tubes were frozen and thawed for four cycles to lyse the cells by transferring from a  $-20^\circ\text{C}$  freezer to a  $37^\circ\text{C}$  water bath. Lysates were centrifuged (Eppendorf) at  $4^\circ\text{C}$  for 30 min at full speed. The supernatants were transferred to clean tubes and kept on ice if the assay was to be performed within 1 h or, otherwise, stored at  $-70^\circ\text{C}$ . The caspase activity assay was performed according to the protocol provided by the supplier. In brief, the reaction components containing caspase substrates were thoroughly mixed with the blank or samples in a 96-well microtiter plate. After incubation at  $37^\circ\text{C}$  for 3 h, the plates were scanned by a fluorescent plate reader to measure the AFC fluorescence at excitation and emission wavelengths of 395 and 525 nm, respectively. The rate of AFC fluorescence signal change was proportional to the enzyme activity. All values were corrected by blank readings and the enzyme activity was normalized to a fixed protein concentration. The activity of individual caspase in samples from control and treated cells at any time point of treatment was measured and compared. The results were expressed as percentage of control, as described in our previous study.<sup>33</sup>

Protein contents were measured by using Bio-Rad assay. The color change of Coomassie brilliant blue G-250 dye (shifted from 465 to 595 nm when binding to protein) was detected by a plate reader, and compared to a standard curve.



## Western blotting of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and p53

Equal aliquots of cell lysates (20  $\mu$ g protein) were boiled in a loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 20% glycerol, 4% SDS and 0.2% bromophenol blue) for 5 min and centrifuged at 9000  $\times$  g for 5 min. The samples were subjected to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were then electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.2% Tween-20) containing 5% dried nonfat milk or BSA for 1–2 h at room temperature. Subsequently, membranes were blotted with one of the antibodies: anti-p21<sup>Waf1/Cip1</sup> (1 : 500), anti-p27<sup>Kip1</sup> (1 : 1000) or anti-p53 (1 : 500), for 1 h (2 h for p53) at room temperature. After three washes in TBST (5 min each), the membranes (for p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>) were incubated with a suitable horseradish peroxidase-conjugated secondary antibody (anti-goat IgG; 1 : 1000) for 1 h at room temperature. There was no necessity for the incubation of a second antibody for p53 detection, since the primary antibody was directly conjugated with POD. The membranes were washed again (3  $\times$  15 min) in TBST and chemiluminescent signals were detected using an ECL system (from Pierce, Rockford, IL, USA). The bands in the exposed films were analyzed by densitometry. For the time-course experiments, results were first expressed as the ratio of optical density readings of treatment and control samples. The ratios at different treatment time points were then expressed as the percentage of zero time for both control and experimental samples.<sup>33</sup>

## Partial cloning of p21<sup>Waf1/Cip1</sup> cDNA in HIT-T15 cells by RT-PCR

Interference with mRNA processing by transfection of siRNA duplexes is a novel and powerful approach to knock down targeted protein in cells.<sup>47</sup> However, this technique requires the complete match of the siRNA nucleotide sequences with those of targeted mRNA. The HIT-T15 cells used in this study are derived from hamster and the cDNA sequence of p21<sup>Waf1/Cip1</sup> in this species is not available. Therefore, we cloned and sequenced a fragment of  $\sim$ 450 nucleotides corresponding to the 5' region of p21<sup>Waf1/Cip1</sup> cDNA from HIT-T15 cells. In brief, total RNA was extracted from 5  $\times$  10<sup>7</sup> HIT cells using TRIZOL reagent (GIBCO BRL), the total RNA (5  $\mu$ g) was reverse transcribed into cDNA in a reaction volume of 20  $\mu$ l using the Superscript Preamplification System (GIBCO BRL). The primers 5'-TAAGGGGAATTGGAGGCAGG-3' and 5'-AGTCTT-CAGGCCTCTCAGGG-3' corresponding to nucleotides 21–41 and 453–472 of rat p21<sup>Waf1/Cip1</sup> mRNA (U24174) were used to amplify p21<sup>Waf1/Cip1</sup> cDNA from HIT cells. The PCR was performed in a reaction of 50  $\mu$ l at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for a total of 40 cycles. The PCR product was cloned into pDrive (Invitrogen) and sequenced using M13 and T7 primers. The sequence obtained consistently from three independent experiments was compared with the p21<sup>Waf1/Cip1</sup> cDNA sequences of other species by the BLAST 2 Program. The sequence identity is 86, 88 and 79% in relation to its rat, mouse and human homologs, respectively.

## Knockdown of p21<sup>Waf1/Cip1</sup> in HIT-T15 cells by the siRNA technique

The p21<sup>Waf1/Cip1</sup> siRNA duplex was annealed by a pair of complementary RNA primers of 21 nucleotides (ordered from Dharmacon Research, Inc., Lafayette, CO, USA) corresponding to the 5'-coding region (137–157) of hamster p21<sup>Waf1/Cip1</sup> cDNA with 2-nucleotide (2'-deoxy) thymidine 3'-overhangs (5'-AAC GGU GGA ACU UCG ACU UUG dTdT-3' and 5'-AAC

AAA GUC GAA GUU CCA CCG dTdT-3'). The selection of siRNA sequences was following the criteria provided by Dharmacon Research, Inc., which are based on established work,<sup>47</sup> including (1) locating the first AA dimmer downstream of 50–100 bases from the start codon; (2) recording the next 19 nucleotides following the AA dimmer; (3) ascertaining the G/C content of the AA-N<sub>19</sub> sequence being 30–70%; and (4) running a BLAST search to ensure that no other genes are targeted by the selected sequence. Another siRNA (5'-AAC GUA CGC GGA AUA CUU CGA dTdT-3') that does not match any gene by BLAST search served as a negative control.

The day before transfection, HIT-T15 cells were plated onto six-well plates in fresh RPMI medium with 10% FBS without antibiotics. Transient transfection of siRNAs was carried out by using Oligofectamine (Invitrogen), following the manufacturer's instructions. In brief, 2.66  $\mu$ g (10  $\mu$ l of 20  $\mu$ M) duplex of p21<sup>Waf1/Cip1</sup> siRNA or control siRNA was mixed with 175  $\mu$ l Opti-MEM (fresh RPMI medium without antibiotics), and then complexed with the mixture of 3  $\mu$ l of Oligofectamine and 15  $\mu$ l Opti-MEM for 20 min at room temperature. The RNA : Oligofectamine complex was diluted with 800  $\mu$ l Opti-MEM to obtain a final volume of 1000  $\mu$ l, which was added to the wells for the transfection of cells. After 8 h, the cells were replenished with 500  $\mu$ l Opti-MEM containing 30% fetal calf serum and incubated for another 18 h. Cells were then treated by MPA (3  $\mu$ g/ml) for 32 h and subsequently harvested for Western blotting of p21<sup>Waf1/Cip1</sup>, measurement of caspase-2 activity and PI staining for assessment of apoptosis, as described above.

## Statistical analysis

All data are presented as the means  $\pm$  S.E.M. for a series of *n* experiments. Statistical analyses were performed by using the *t*-test or one-way analysis of variance (ANOVA), followed by the appropriate *post hoc* comparison. Group differences with *P* < 0.05 were considered statistically significant.

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