Published in final edited form as:
Oncogene. 2009 October 15; 28(41): 3619–3630. doi:10.1038/onc.2009.225.

Regulation of p53 expression, phosphorylation and sub-cellular localisation by a G-protein coupled receptor

Lev Solyakov[1,*], **Emre Sayan**[2,*], **Joan Riley**[#], **Amy Pointon**[#], and **Andrew B Tobin**[1]

[1]Department of Cell Physiology and Pharmacology, University of Leicester, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN, UK

[2]Department of Cancer Studies and Molecular Medicine, University of Leicester, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN, UK

[#]MRC Toxicology Unit, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN

Abstract

G-protein coupled receptors (GPCRs) have been extremely successful drug targets for a multitude of diseases from heart failure to depression. This super-family of cell surface receptors have not, however, been widely considered as a viable target in cancer treatment. In the current study we demonstrate that a classical $G_{q/11}$-coupled GPCR, the M_3-muscarinic receptor, was able to regulate apoptosis via receptors that are endogenously expressed in the human neuroblastoma cell line SH-SY5Y and when ectopically expressed in Chinese hamster ovary (CHO) cells. Stimulation of the M_3-muscarinic receptor was shown to inhibit the ability of the DNA-damaging chemotherapeutic agent, etoposide, from mediating apoptosis. This protective response in CHO cells correlated with the ability of the receptor to regulate the expression levels of p53. In contrast, stimulation of endogenous muscarinic receptors in SH-SY5Y cells did not regulate p53 expression but rather was able to inhibit p53 translocation to the mitochondria and p53 phosphorylation at serine 15 and 37. This study **suggests the possibility that** a GPCR can regulate the apoptotic properties of a chemotherapeutic DNA-damaging agent by regulating the expression, sub-cellular trafficking and modification of p53 in a manner that is in part dependent on the cell type.

Keywords

Apoptosis; p53; muscarinic; G-protein coupled receptor; M_3-muscarinic; phosphorylation; mitochondria; SH-SY5Y

Introduction

The wide involvement of G-protein coupled receptors (GPCRs) in biological responses coupled to the fact that these receptors show very distinct tissue distributions and are activated by specific ligands have made this receptor super-family an ideal candidate for pharmacological intervention in a large number of diseases (Klabunde and Hessler, 2002). Despite this success, targeting GPCRs in diseases where programmed cell death (apoptosis) is a feature has been largely over looked. Prevalent among such diseases is cancer where apoptosis is suppressed, primarily by liaisons in the p53 pathway (Vousden and Lu, 2002), and where chemotherapeutic intervention is often directed to inducing apoptosis in the cancerous cells .

Corresponding author: Prof. Andrew B. Tobin, Tel: 0116 2522935, Fax: 0116 2231405, Email: tba@le.ac.uk.
*Authors contributed equally

Europe PMC Funders Author Manuscripts

It has been known for some time that GPCRs can influence apoptosis in both a positive and negative manner depending on the cell type and receptor subtype being investigated (Chen *et al.*, 1999; De Sarno *et al.*, 2005; DeFea *et al.*, 2000; Diao *et al.*, 2000; Fang *et al.*, 2000; Murga *et al.*, 1998; Tobin and Budd, 2003; Vichalkovski *et al.*, 2005; Zhu *et al.*, 2001). The ability of GPCRs to regulate apoptosis is likely due to the plethora of signalling pathways activated by these receptors, many of which are known to converge on apoptotic pathways (Dorsam and Gutkind, 2007; Marinissen and Gutkind, 2001). Thus, GPCRs are able to potently activate the PI-3 kinase/Akt pathway as well as the mitogen-activated protein (MAP) kinase pathways, ERK-1/2, Jun-kinase and p38, and thereby influence apoptosis (Fang et al., 2000; Murga et al., 1998; Zhou et al., 2002; Zhu et al., 2001). Similarly, receptors coupled to calcium mobilisation, protein kinase C (PKC) activation and cyclic-AMP production have all been shown to either promote or inhibit apoptosis (Dale *et al.*, 2000; Diao *et al.*, 2000; Kwon *et al.*, 2001; Tobin and Budd, 2003).

In terms of cancer treatment many laboratories have focused on the role played by the transcription factor p53 which appears to mediate apoptosis induced by many of the commonly used DNA-damaging chemotherapeutic agents (for discussion see (Brown and Attardi, 2005)). Furthermore, in approximately 50% of human tumours p53 inactivating mutations are found and in those tumours where there is no mutation at the p53 locus there is often a lesion in the pathways that regulate p53 expression or sub-cellular localisation (Haupt and Haupt, 2006; Vogelstein *et al.*, 2000). In light of the ability of GPCRs to regulate the apoptotic process we wanted to test if the signalling potential of these receptors could extend to the regulation of p53 under conditions where apoptosis was induced by a chemotherapeutic DNA damaging agent.

Our work has centred on the M_3-muscarinic receptor which is a classical $G_{q/11}$-coupled GPCR that activates the PLC/calcium mobilisation pathway (van Koppen and Kaiser, 2003). We and others have established that the M_3-muscarinic receptor is able to protect cells from apoptosis in transfected cell lines (Budd *et al.*, 2003; Tobin and Budd, 2003), the human neuroblastoma cell line SH-SY5Y (De Sarno et al., 2003; De Sarno et al., 2005), cortical neurons (Koh et al., 1991) and cerebellar granule neurons (Yan et al., 1995). Here we use the fact that the M_3-muscarinic receptor is a GPCR subtype able to regulate the process of apoptosis seen in response to DNA-damage (Budd *et al.*, 2003; Tobin and Budd, 2003) to test the notion that GPCRs were able to influence the cellular function of p53. To do this we use two cell lines a Chinese hamster ovary cell line transfected with the human M_3-muscarinic receptor (CHO-m3 cells) and the human neuroblastoma cell line, SH-SY5Y, that endogenously expresses the M_3-muscarinic receptor.

Results

The M_3-muscarinic receptor regulates the expression of p53 protein in CHO-m3 cells

Treatment of CHO-m3 cells with etoposide for 16 hours resulted in a ~2.5-fold increase in caspase activity over control levels. Stimulation of the M_3-muscarinic receptor using the full agonist methylcholine significantly reduced caspase activation by 69.4 ±8.9% ($p<0.01$, $n=6$) (Fig.1A); an observation consistent with previous studies (Budd *et al.*, 2004; Tobin and Budd, 2003). The effect of methylcholine was blocked by the muscarinic specific antagonist, atropine (Fig 1A). The ability of M_3-muscarinic receptor stimulation to reduce etoposide-mediated caspase activation correlated with changes in the expression levels of p53. Levels of p53 increased in CHO-m3 cells challenged with etoposide and this was attenuated by the stimulation of the M_3-muscarinic receptor (Fig 1B).

In our studies p53 appears as a double band in Western blots (Fig 1B). Although the reason for this is unclear it is possible that it either reflects differential phosphorylation status of

p53 (Hu et al., 1999; Ullrich et al., 1992) or different p53 isoforms resulting from differential splicing, polymorphisms or alternative promoter usage (Bourdon, 2007; Courtois *et al.*, 2004; Dumont *et al.*, 2003).

si-RNA specific to p53 reduced the induction of p53 expression following etoposide treatment by ~50% (Fig. 1C). This correlated with a similar reduction in etoposide-induced caspase 3 activity (Fig 1C). Thus, indicating that caspase 3 activation following etoposide treatment was mediated via an increase in p53 expression levels.

The ability of the M_3-muscarinic receptor to down regulate p53 and protect cells from apoptosis correlated with the ability of the receptor to prevent the activation of p53 responsive genes. RT-PCR analysis demonstrated that transcription of the p53 responsive genes Bax, Mdm2 and NOXA were significantly increased following etoposide treatment (Fig 1D). Stimulation of the M_3-muscarinic receptor reduced transcription of these genes to basal levels (Fig 1D) in a manner that correlated with the ability of the receptor to protect from apoptosis.

We tested whether a short period of muscarinic receptor treatment would be sufficient to down-regulate p53 levels in CHO-m3 cells resulting from prolonged treatment with etoposide. In control experiments we found that four hours treatment with etoposide resulted in an increase in p53 expression levels and that this was attenuated by a simultaneous four hour treatment with methylcholine (Fig. 1E). If methylcholine stimulation was stopped after four hours with the antagonist atropine (10μM; a concentration sufficient to completely block muscarinic receptor signalling (Budd et al., 2004)) and the treatment with etoposide continued for a further 12 hours the attenuation of p53 levels was still evident (Fig. 1E). These data indicate that transient receptor stimulation was sufficient to suppress etoposide mediated p53 expression levels. Interestingly, the suppression of p53 levels by the muscarinic receptor was not due to the ability of the receptor to enhance p53 degradation. This was demonstrated by inhibiting new protein synthesis using cyclohexamide and monitoring the loss of p53 over time. Here etoposide treatment significantly decreased the rate of p53 degradation in a manner that was not affected by the presence of methacholine (Fig 1F).

Further RT-PCR studies revealed that the ability of the M_3-muscarinic receptor to decrease p53 protein expression levels in CHO-m3 cells was not due to an ability to decrease transcription of p53 **(**supplementary Fig.S1**)**. Since, DNA-damage had previously been reported to both stabilisation and activate p53 through a process that is in part due to phosphorylation of specific serine residues at the N-terminus of p53 (Chao et al., 2000; Chehab et al., 1999; Meek, 1999; Shieh et al., 1997; Siliciano et al., 1997; Unger et al., 1999) the phosphorylation status of p53 was investigated. Of particular importance is serine-15 where phosphorylated has been reported to increase p53 stability via disruption of Mdm2 binding (Shieh et al., 1997; Siliciano et al., 1997). We tested here if muscarinic receptor stimulation was able to regulate the level of p53 phosphorylation at serine-15 using a phospho-specific antibody. Treatment with etoposide resulted in a robust phosphorylation of p53 on serine-15 (Fig. 1G). Stimulation with methylcholine resulted in a decrease in serine-15 phosphorylation which correlated closely with the decrease in total p53 levels (Fig. 1G). Hence, in these experiments we found no evidence that p53 de-phosphorylation at serine-15 preceded p53 down-regulation. Similarly we tested the possibility that phosphorylation at serine-392 (Fig. 1H) was changed by M_3-muscarinic receptor stimulation. However, although levels of phosphorylation did decrease, this correlated with decreases in total p53 levels. Hence, it was again not possible to conclude whether p53 de-phosphorylation preceded receptor mediated p53 degradation.

Stimulation of the M_3-muscarinic receptor protects SH-SY5Y cells against etoposide-induced caspase activation

Consistent with previous reports (De Sarno et al., 2003; De Sarno et al., 2005) we show here that the M_3-muscarinic receptor endogenously expressed in the human neuroblastoma cell line SH-SY5Y was able to protect cells from etoposide induced cell death. Treatment with etoposide (25μM) for 4 and 8 hours resulted in 6-8 fold increase in caspase activity which was significantly reduced by methylcholine (90.2 ± 0.1% ($P<0.001$, n=5) and 59.3 ± 0.7% ($P<0.05$, n=3), respectively (Fig 2A)). Furthermore, muscarinic receptor stimulation was able to attenuate caspase-mediated PARP cleavage in response to etoposide treatment (Fig 2B), caspase 3 and caspase 9 processing together and cytochrome C release into the cytoplasm (Fig 2C). We did not, however, observe any changes in caspase 8 processing nor cleavage of the caspase 8 substrate Bid (Fig 2C).

Time course studies were conducted where M_3-muscarinic receptor stimulation was stopped by addition of the antagonist atropine (10μM) at various times during the 4 hour challenge with etoposide. These studies revealed that caspase activation measured after 4 hours treatment with etoposide was reduced by 66.7 ± 0.3% ($P<0.05$, n=4) after a 30 min stimulation of M_3-muscarinic receptors initiated at the start of the etoposide treatment (Fig 2D). These data suggest that a transient stimulation of M_3-muscarinic receptor signalling has is sufficient to protect SHSY5Y cells from a prolonged exposure to etoposide.

Pharmacological inhibitors to the PLC/calcium mobilisation pathway (U73122 and dantrolene), PKC (Ro31-8220) and MEK (PD98059) had no affect on the ability of methylcholine to inhibit caspase activation (supplementary Fig S2). Furthermore, a number of other inhibitors including the CK1 inhibitor D-4476 **(**supplementary Fig S3A**)** JNK inhibitor I (5μM), the CaM-kinase II inhibitor KN-93 (10μM), selective PKA inhibitors KT 5720 (1μM) and H-89 (1μM) similarly had no affect on the M_3-muscarinic anti-apoptotic response in SH-SY5H cells **(**supplementary Fig3B**).** In addition, the PI3-kinase inhibitors LY294002 (10μM) and wortmannin (100nM) and the inhibitor of mTOR, rapamycin, were unable to affect M_3-muscarinic receptor-mediated protection of SH-SY5Y cells (supplementary Fig S4). However, the broad spectrum protein kinase inhibitor HA1077 was able to inhibit the action of methylcholine (supplementary Fig. S5). This has been previously attributed to the ability of this compound to inhibit Rho-kinase (De Sarno et al., 2005). However, the Rho-kinase specific inhibitor, Y-27632 (Chitaley et al., 2001), was unable to affect the muscarinic response when used at a concentration of 10μM (supplementary Fig S5). This would suggest that the Rho-kinase pathway is not directly involved in the M_3-muscarinic receptor-mediated protection mechanism but that protein phosphorylation is important.

M_3-muscarinic receptors inhibit apoptosis in SH-SY5Y cells but do not change the level of p53 expression

In light of the ability of the M_3-muscarinic receptor to regulate the expression levels of p53 following a challenge with etoposide in CHO-m3 cells we tested if muscarinic receptor stimulation could change the expression levels of p53 in SH-SY5Y cells. Etoposide treatment of SH-SY5Y cells for two, four and eight hours resulted in a substantial increase in p53 expression that was not significantly affected by co-stimulation with methylcholine (Fig 3A). Hence, in contrast to CHO-m3 cells, M_3-muscarinic receptors do not appear to regulate the expression levels of p53 in SH-SY5Y cells.

We next confirmed that the apoptotic response observed in SH-SY5Y cells following etoposide treatment was mediated by elevated levels of p53. SH-SY5Y cells were transfected with siRNA duplex directed against p53 or with scrambled control. The cells

were then challenged with etoposide. In control cells etoposide treatment resulted in an increase in the expression levels of p53 that correlated with an increase in caspase 3 processing and the cleavage of the caspase 3 substrate PARP (lane 2, Fig 3B). In siRNA treated cells the induction of p53 was significantly reduced (by ~50%). In these cells caspase 3 processing and PARP cleavage were similarly reduced (lane 6, Fig 3B). These data are consistent with p53 mediating etoposide-induced apoptosis in SH-SY5Y cells.

A large body of work from other laboratories have established that p53-mediate apoptosis is primarily attributable to the ability of p53 to transcriptionally regulate pro-apoptotic genes such as Puma, NOXA and Bax (Nakano and Vousden, 2001; Oda *et al.*, 2000; Yu *et al.*, 1999). In the current study a four hour treatment with etoposide did increase the transcription of the p53 responsive genes Bax and Mdm2 (Fig. 4A). However, Western blots of the proteins demonstrated that there was a small increase in the expression of Mdm2 and no significant increase in the level of Bax protein (Fig 4B). Thus, despite evidence that p53 was responsible for etoposide-induced apoptosis (Fig 3B) this did not appear to be linked with a substantial change in the expression of pro-apoptotic p53 responsive genes (Fig 4B). Furthermore, the ability of muscarinic receptor stimulation to protect from etoposide-induced apoptosis was not correlated with a change in the transcription of p53-reponsive genes (Fig 4A). It seems likely, therefore, that although p53 is the mediator of apoptosis induced by etoposide this is not through an up regulation of p53 responsive gene expression. Consistent with this conclusion was the fact that the pharmacological inhibitor of p53-mediated transactivation, pifithrin-α (Komarov et al., 1999) had no affect on etoposide-mediated caspase activity (Fig 4C).

That p53 was the mediator of the increase in caspase activity following etoposide treatment of SH-SY5Y cells was, however, evident firstly by the effects of p53 siRNA (see; Fig 3B) and secondly by the fact that suppression of p53 activity using pifithrin-μ (Strom et al., 2006), prevented etoposide-mediated caspase activation (Fig 4D). Pifithrin-μ has been reported to inhibit p53 translocation to the mitochondria and in this way prevent apoptosis resulting from the direct action of p53 on the mitochondria (Strom et al., 2006). In our hands pifithrin-μ prevented the elevation of total p53 in response to DNA-damage and this correlated with a total protection from etoposide induced apoptosis (Fig 4D-insert).

M_3-muscarinic receptors inhibit translocation of p53 to the mitochondria

Based on reports demonstrating that rapid apoptosis induced within the first few hours of DNA-damage can be mediated by the direct action of p53 on the mitochondria (Erster et al., 2004; Marchenko et al., 2000) we tested the possibility that the muscarinic receptor protective response might correlate with changes in p53 translocation to the mitochondria. We found that etoposide treatment resulted in a rapid rise in the expression levels of p53 in the nuclear, cytosolic and mitochondrial fractions in SH-SY5Y cells (Fig 5). M_3-muscarinic receptor stimulation had no significant affect on the levels of etoposide-induced p53 levels in the nucleus or cytoplasm but did significantly decrease the levels of p53 in the mitochondria (Fig 5).

M_3-muscarinic receptors regulate the phosphorylation status of p53

It is well documented that the phosphorylation status of p53 is up-regulated following DNA damage and that this modification contributes to the stabilisation and activation of p53 (Chao et al., 2000; Chehab et al., 1999; Meek, 1999; Shieh et al., 1997; Siliciano et al., 1997; Unger et al., 1999). To test if GPCR activity could regulate the phosphorylation status of p53 in SH-SY5Y cells, phospho-specific antibodies were used in Western blots of p53 contained in the nuclear, cytoplasmic and mitochondrial fractions. Following a 4 hour treatment with etoposide the phosphorylation of p53 at residues 6, 9, 15, 20, 37 and 392

increased in the nuclear, cytoplasmic and mitochondrial fractions (Fig 6A). Muscarinic receptor stimulation did not affect the phosphorylation of p53 in nuclear fractions (Fig6A). Similarly, mitochondrial p53 phosphorylation did not appear to be affected by muscarinic receptor stimulation since the apparent decrease in the phosphorylation status of p53 following receptor activation observed in Fig 6A could be accounted for by the decrease in total mitochondrial p53. However, muscarinic receptor stimulation did significantly reduce the phosphorylation status of cytoplasmic p53 at residues 15 and 37 (Fig 6A orange boxes and Fig 6B).

Discussion

Our study demonstrates the ability of a $G_{q/11}$-coupled GPCR to regulate caspase activation following DNA-damage. In CHO-m3 cells, where apoptosis proceeds following a prolonged exposure to etoposide, the muscarinic anti-apoptotic response correlates with a decrease in p53 expression levels. In contrast, etoposide-mediated caspase activation in SH-SY5Y cells is rapid and the muscarinic protective response does not correlate with a change in the overall level of p53 expression but rather is associated with a decrease in the translocation of p53 to the mitochondria. Furthermore, muscarinic receptor stimulation regulates the phosphorylation status of p53 in the cytoplasm of SH-SY5Y cells. This study, therefore, demonstrates the previously unknown capacity of a GPCR to regulate p53 at a number of different levels and in a cell type dependent manner.

Decreasing p53 expression levels in CHO-m3 cells, via siRNA, results in a reduction in etoposide-mediated caspase activation thus supporting the conclusion that etopoisde promotes apoptosis in CHO-m3 cells by elevating p53 expression. In light of this it is likely that the mechanism by which M_3-muscarinic receptors protect CHO-m3 cells from apoptosis is via the ability of this receptor to decrease p53 expression levels. This is important in the context of previous studies where the anti-apoptotic response of this receptor subtype was shown to correlate with an up-regulation of Bcl-2 protein (Tobin and Budd, 2003) a gene that is under negative control by p53 (Miyashita et al., 1994). This provides a further explanation for the anti-apoptotic properties of the M_3-muscarinic receptor subtype in CHO-m3 cells which both decreases p53 levels (this study) and correspondingly increases Bcl-2 levels (Tobin and Budd, 2003). The mechanism by which the M_3-muscarinic receptor is able to regulate p53 expression in CHO-m3 cells is, however, unknown. Previous studies have eliminated a role for PLC/calcium signalling, the MAP kinase pathways as well as the PI3-kinase pathway (Budd *et al.*, 2003; Tobin and Budd, 2003). However, a polybasic motif contained at the C-terminal tail of the receptor has been shown to be essential for the ability of the receptor to couple to the anti-apoptotic pathway in this cell type (Budd *et al.*, 2003; Tobin and Budd, 2003). In the current study the possibility that the receptor was able to regulate the phosphorylation of p53 at serine-15 and thereby regulate p53 stability was investigated. However, it appears that changes in p53 serine-15 and serine-392 phosphorylation did not precede p53 down regulation. It is possible that M_3-muscarinic receptor signalling is able to regulate p53 phosphorylation in CHO-m3 cells but the resolution of the Western blot techniques used here is not sufficiently high to detect these changes. Clearly further studies are required to define the mechanism by which GPCRs can regulate p53 expression.

In contrast to the situation in CHO-m3 cells, the mechanism by which M_3-muscarinic receptors reduce caspase activity in the human neuroblastoma cell line SH-SY5Y appears not to be centred on the control of p53 expression. In this cell line caspase activity in response to DNA-damage is rapid and does not correlate with a change in pro-apoptotic p53 target genes. Previous studies have determined that caspase activation in SH-SY5Y cells in response to etoposide is mediated by the action of p53 (Cui et al., 2002). Consistent with this

we find here the p53 inhibitor, pifithrin-μ, blocked the ability of etoposide to mediate caspase activation. Pifithrin-μ has been reported to prevent p53 mitochondrial translocation (Strom et al., 2006) but in our hands this inhibitor prevents etoposide-mediated increases in total p53 levels. Furthermore, knock down of p53 expression levels using siRNA correspondingly reduced the apoptotic response to etoposide confirming that p53 was the mediator of the apoptotic response in SH-SY5Y cells.

Importantly, pifithrin-α, an inhibitor of p53 transactivation (Komarov et al., 1999), did not prevent etoposide-mediated caspase activation. Furthermore, although etoposide did induce p53-mediated transcription of pro-apoptotic proteins this was not reflected in an increase in protein expression levels seen at the short time points of incubation used (i.e. 4 hours). Thus, although DNA-damage induces caspase activation in SH-SY5Y cells via a p53-dependent mechanism, this does not appear to require an increase in the expression levels of p53 responsive genes. One possibility is that p53 is able to mediate its affects via a direct action on the mitochondria. This phenomenon has been reported previously to account for etoposide-mediated apoptosis in SH-SY5Y cells (Sayan et al., 2006) and has also been reported in a number of other model cell lines (Chipuk and Green, 2003; Moll *et al.*, 2005) where it has been suggested to account for the first wave of p53-mediated apoptosis which precedes a later sustained period of apoptosis mediated by p53 gene transcription (Erster et al., 2004).

The importance of the fact that etoposide-mediated apoptosis in SH-SY5Y cells may be a direct action of p53 on mitochondria is borne out in the observations that muscarinic receptor stimulation prevents cytochrome C release into the cytoplasm, prevents caspase 3 and 9 processing and reduces the translocation of p53 to the mitochondria. In the context of these findings it is likely that the ability of the M_3-muscarinic receptor to protect SH-SY5Y cells from etoposide-induced apoptosis is centred on the ability of the receptor to prevent the translocation of p53 to the mitochondria.

As is the case for muscarinic receptor mediated effects on p53 in CHO-m3 cells, the signalling pathway employed by the muscarinic receptor to regulate p53 function in SH-SY5Y cells is unclear. Inhibitors of Rho-kinase did not provide evidence for the involvement of this pathway since the specific Rho-kinase inhibitor Y-27632 (Chitaley et al., 2001), did not prevent the muscarinic anti-apoptotic response. Similarly, pharmacological inhibitors of PI-3 kinase, PLC/calcium signalling, MAP kinases, CK1, mTOR, PKA and PKC did not identify any candidate pathways.

Cellular stress results in hyper-phosphorylation of p53 which is known to contribute to the both stabilisation and transcriptional activity of p53 (Bode and Dong, 2004; Brooks and Gu, 2003). Consistent with this we show here that etoposide treatment resulted in increased phosphorylation at serine residues 6, 9, 15, 20, 37 and 392 on p53. Moreover, muscarinic receptor stimulation decreased the phosphorylation status of p53 only at positions 15 and 37 and only in p53 located in the cytoplasmic fraction. This is the first demonstration of a GPCR regulating the phosphorylation status of p53 and may provide an explanation for the anti-apoptotic properties of the M_3-muscarinic receptor since phosphorylation at these residues has been shown to be important for p53-mediated apoptosis (Li et al., 2006; Shieh et al., 1997).

Both functionally and mechanistically phosphorylation at serine 15 and 37 appear to be linked. Phosphorylation at serine 15 and 37 disrupts MDM2 binding and thereby stabilises p53 in response to DNA damage (Bean and Stark, 2001; Shieh *et al.*, 1997). Furthermore, phosphorylation at these two sites contributes to increasing p53 transcriptional activity by promoting the interaction with histone acyltransferases (CBP/p300, PCAF/p300) resulting in

C-terminal acetylation of p53 and thereby specific DNA binding (Lambert et al., 1998; Sakaguchi et al., 1998). *In vitro* studies have determined that p53 is phosphorylated at serine 15 and 37 by the same protein kinases, DNA-dependent protein kinase (Shieh et al., 1997) and ATR (Tibbetts et al., 1999), both of which respond to DNA damage. In recent studies it has been suggested that phosphorylation at these sites may be regulated by the action of the protein phosphatase, PP-1, which can promote cell survival by dephosphorylation of serine 15 and 37 (Li et al., 2006). It is therefore of considerable interest that M_3-muscarinic receptor stimulation can regulate the level of phosphorylation of p53 at these two key phospho-acceptor sites.

It is tempting to speculate that the phosphorylation status of cytoplasmic p53 may contribute to the translocation of p53 to the mitochondria. Recent studies have suggested that serine 15 phosphorylation can promote p53 mitochondrial translocation and interaction with Bcl-2 and Bcl-XL (Park et al., 2005). However, other studies have not been able to correlate phosphorylation and acetylation of p53 with mitochondrial targeting (Nemajerova et al., 2005). Hence, it is not currently possible to determine if the ability of the receptor to reduce serine 15 and 37 phosphorylation of cytoplasmic p53 observed in this study is linked with the ability of muscarinic receptor stimulation to reduce p53 translocation to the mitochondria.

Our studies suggest the possibility that a GPCR can regulate apoptosis induced by DNA damage through direct action on p53. We would predict that this principle can be extended to GPCRs other than the M_3-muscarinic receptor, particularly in light of the fact that a large number of GPCRs can regulate the apoptotic process (Budd *et al.*, 2003; Budd *et al.*, 2004; Chen *et al.*, 1999; Dale *et al.*, 2000; De Sarno *et al.*, 2003; De Sarno *et al.*, 2005; DeFea *et al.*, 2000; Diao *et al.*, 2000; Fang *et al.*, 2000; Koh *et al.*, 1991; Murga *et al.*, 1998; Tobin and Budd, 2003; Vichalkovski *et al.*, 2005; Yan *et al.*, 1995; Zhu *et al.*, 2001). In particular recent studies on the lysophosphatidic acid receptor has demonstrated that this GPCR subtype can regulate the expression levels of p53 in cancer cells (Murph *et al.*, 2007). Since GPCRs provide a well established pharmaceutical target it may be possible to specifically target GPCRs on cancer cells to regulate p53-dependent processes. Depending on the GPCR receptor subtype (and almost certainly the cancer type) we would speculate that a GPCR agonist or antagonist could be used to augment the action of a chemotherapeutic agent. In the example of the M_3-muscarinic receptor an antagonist would have to be used to prevent autocrine/paracrine activation of this anti-apoptotic pathway. That such autocrine loops exist in tumours has recently been highlighted in a report where release of acetylcholine from small cell lung cancer cells was shown to promote tumour growth through the activation of the M_3-muscarinic receptor subtype in a fashion that is inhibited by muscarinic receptor antagonists (Song et al., 2007).

Materials and Methods

Materials

Anti-cytochrome C antibody was obtained from BD Pharmingen (BD Biosciences, Oxford, UK). Primary antibodies against-tubulin, p53 (D0-1), NOXA and Mdm2 were purchased from Santa Cruz Biotechnology, Inc. (Autogen Bioclear UK Ltd, Wiltshire, UK). Primary antibodies against PUMA, PARP (cleaved fragment), caspase-9 (Asp330, cleaved fragment), caspase-3 (cleaved fragment), and all phospho-p53 specific antibodies were purchased from Cell Signaling Technology. Anti-mouse and anti-rabbit HRP-conjugates and U73122 were obtained from Sigma (Sigma-Aldrich Company Ltd. Poole, Dorset, UK). D-4476, JNK inhibitor I, KT-5720, H-89, RO-318220, dantrolene, pifithrin-α, pifithrin-μ and PD98059 were purchased from Calbiochem. KN-93 from Merck and LY294002 from Tocris. All other chemicals and reagents were obtained from the usual commercial sources.

Cell Culture and drug treatments

Chinese hamster ovary cells stably transfected with the human M_3-muscarinic receptor were cultured in α-MEM supplemented with 10% fetal bovine serum, 2.5 μg/ml of fungizone, 50 U/ml of penicillin, 50 μg/ml of streptomycin and 250 μg/ml G-418 sulphate. The human neuroblastoma SH-SY5Y cells were cultured in 10cm Petri dishes or 175 cm^2 flasks and maintained in MEM media (Earl's modified), supplemented with 5% fetal bovine serum, 5% newborn calf serum, 2 mM L-glutamine, 2.5 μg/ml of fungizone, 50 U/ml of penicillin and 50 μg/ml of streptomycin.

Apoptosis was induced by treating cells, reaching 50-60% confluency, with etoposide at the indicated concentrations. The muscarinic agonist, methylcholine was applied for the indicated time periods (see results section and figures) and where indicated the stimulation was stopped by addition of atropine (10μM), a specific antagonist for muscarinic receptors.

In experiments where cyclohexamide was used cells were treated with or without etoposide in the presence or absence of methacholine for 16 hours. Cyclohexamide (20μg/ml) was then added for the indicated times and the reaction stopped by addition of lysis buffer (50mM HEPES, pH 7.4, 0.15M NaCl, 2mM EDTA, 1mM DTT, 1% CHAPS) Lysates were then used in caspase assays as described below.

Caspase-3 activity assay

Following appropriate treatment, cells were harvested in PBS-EDTA buffer, washed and cell pellets placed on ice and lysed for 10 min in lysis buffer. Cell lysates were cleared by centrifugation at 14,000 rpm for 3 min, 4°C, and the supernatant fraction was used for caspase-3 activity assay. Aliquots of the cell lysate containing equal amount of proteins (normally 200μg per assay) were incubated in reaction mixture containing Ac-DEVD-*p*NA (final concentration 200μM) and 10mM DTT for 3 hours at 37°C. Cleavage of the Ac-DEVD-*p*NA substrate was measured at 405 nm in plate reader using 96-well plates.

Cells fractionation

SH-SY5Y cells were cultured in 175 cm^2 flasks until ~ 70-80% confluency. Following treatment cells were harvested in PBS-EDTA buffer, washed once with ice-cold PBS and resuspended in ice-cold hypotonic buffer (20mM HEPES, pH 7.4, 250mM sucrose, 5mM $MgCl_2$, 10mM KCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF and protease inhibitor cocktail (Sigma)). Following 15 min incubation on ice the cell suspension was passed 8 times through a 27 gauge needle and centrifuged at 750xg for 10min. The pellet was discarded and the supernatant fraction was centrifuged again at 10,000xg for 10 min. The final pellet was taken as mitochondrial fraction while the supernatant as cytosolic one. Samples were resuspended in Laemmli buffer, run over 10% SDS gels and analysed by Western blotting as above. To purify the nuclear fractions, SH-SY5Y cells were maintained and treated in the same manner as it was done for mitochondria fractionation. The cells were harvested, washed in ice-cold PBS and lysed on ice for 5 min in nuclear-lysis buffer (Sigma, NUC-201 nuclei isolation kit). To purify the nuclei, the cell lysates were further centrifuged over 1.8M sucrose cushion solution in accordance with the manual instruction. Isolated nuclei were then analysed by Western blotting.

p53 siRNA treatment

For each treatment (whether CHO-m3 or SHSY-5Y cells), 50pmol of p53 targetting siRNA (Ambion siRNA ID: s605) or control siRNA (Ambion, #AM4611) was transfected in to 1.5×10^6 cells by nucleofection (Amaxa nucleofector, Cologne, Germany) using buffer V, program A023. 48 hours after transfection, cells were treated with etoposide and/or methylcholine as described above.

Real time–PCR (RT–PCR)

Total RNA was isolated using Trizol reagent (Sigma). RNA (100 ng) was reverse-transcribed using Superscript III (Invitrogen) and the resulting cDNA was used for amplification with SYBR® green mastermix (Applied Biosystems). Reactions were carried out on an ABI PRISM® 7700 RT–PCR machine using optimized primers. Primers for RT–PCR were designed to cross exon-exon boundaries to eliminate the detection of any contaminating genomic DNA using Primer Express® software v2.0 (Applied Biosystems). The sequences used were: mmu-Bax forward, TGGAGCTGCAGAGGATGATTG; mmu-Bax reverse, GCTGCCACTCGGAAAAAGAC; mmu-Puma forward, GGGTCCCCTGCCAGATTT; mmu-Puma reverse, GCAGGAGTCCCATGATGATGA; mmu-Mdm2 forward, TCTACAGGGACGCCATCGA, mmu-Mdm2 reverse, GATCCAACCAATCACCTGAATGT; mmu-Noxa forward, GATTTGCGATTGGGATGCA; mmu-Noxa reverse, GCACACTCGACTTCCAGCTACTT; hsa-Bax forward, TGGAGCTGCAGAGGATGATTG, hsa-Bax reverse, GCTGCCACTCGGAAAAAGAC; hsa-Puma forward, GGGTCCCCTGCCAGATTT; hsa-Puma reverse, GCAGGAGTCCCATGATGATGA; hsa-Mdm2 forward, TCTACAGGGACGCCATCGA; hsa-Mdm2 reverse GATCCAACCAATCACCTGAATGT, hsa-Noxa forward, GATTTGCGATTGGGATGCA; hsa-Noxa reverse, GCACACTCGACTTCCAGCTACTT. ACAGCTTTGAGGTTCGTGTT; p53 forward, TCAGTCTGAGTCAGGCCC; p53 reverse.

The expression level of the gene of interest was normalized to that of TATA box binding protein in all samples expect for analysis of p53 that was normalised to actin. Relative quantification of gene expression was performed with the comparative cycle threshold method (Applied Biosystems, User Bulletin no 2, 1997).

p53 status of cell lines used—SH-SY5Y cells contain wild type p53 (Paulsen et al., 2006). The CHO-m3 cell line used was derived from CHO-K1 cells which are described to contain a mutant of p53 at codon 211 (T211K) (Hu et al., 1999). Point mutations at this codon are observed very rarely in primary human tumors (41 reported cases in more than 27,000 identified p53 mutations, see p53 mutation database, http://p53.free.fr/Database/p53_database.html.) indicating that this mutation is not significant for tumour biology. A recent study revealed that the codon 211 mutants of p53 have subtly different transactivator properties than wild type receptor (Kakudo et al., 2005). It is, therefore, possible that the effects of this mutation mean that high concentrations of etoposide (e.g. 250μM) are necessary to obtain caspase activation in CHO-m3 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the Wellcome Trust for their support (Grants 073480 and 047600).

Abbreviations

CHO	Chinese hamster ovary
GPCR	G-protein coupled receptor
ERK	extracellular regulated protein kinase
MAP kinase	mitogen activated protein kinase

PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C

References

Bean LJ, Stark GR. Phosphorylation of serines 15 and 37 is necessary for efficient accumulation of p53 following irradiation with UV. Oncogene. 2001; 20:1076–84. [PubMed: 11314044]

Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer. 2004; 4:793–805. [PubMed: 15510160]

Bourdon JC. p53 and its isoforms in cancer. Br J Cancer. 2007; 97:277–82. [PubMed: 17637683]

Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol. 2003; 15:164–71. [PubMed: 12648672]

Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. Nat Rev Cancer. 2005; 5:231–7. [PubMed: 15738985]

Budd DC, McDonald J, Emsley N, Cain K, Tobin AB. The C-terminal tail of the M3-muscarinic receptor possesses anti-apoptotic properties. J Biol Chem. 2003; 278:19565–73. [PubMed: 12649280]

Budd DC, Spragg EJ, Ridd K, Tobin AB. Signalling of the M3-muscarinic receptor to the anti-apoptotic pathway. Biochem J. 2004; 381:43–9. [PubMed: 15068397]

Chao C, Saito S, Anderson CW, Appella E, Xu Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci U S A. 2000; 97:11936–41. [PubMed: 11035798]

Chehab NH, Malikzay A, Stavridi ES, Halazonetis TD. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci U S A. 1999; 96:13777–82. [PubMed: 10570149]

Chen CK, Burns ME, Spencer M, Niemi GA, Chen J, Hurley JB, et al. Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. Proc Natl Acad Sci U S A. 1999; 96:3718–22. [PubMed: 10097103]

Chipuk JE, Green DR. p53's believe it or not: lessons on transcription-independent death. J Clin Immunol. 2003; 23:355–61. [PubMed: 14601643]

Chitaley K, Wingard CJ, Clinton Webb R, Branam H, Stopper VS, Lewis RW, et al. Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. Nat Med. 2001; 7:119–22. [PubMed: 11135626]

Courtois S, de Fromentel CC, Hainaut P. p53 protein variants: structural and functional similarities with p63 and p73 isoforms. Oncogene. 2004; 23:631–8. [PubMed: 14737098]

Cui H, Schroering A, Ding HF. p53 mediates DNA damaging drug-induced apoptosis through a caspase-9-dependent pathway in SH-SY5Y neuroblastoma cells. Mol Cancer Ther. 2002; 1:679–86. [PubMed: 12479364]

Dale LB, Bhattacharya M, Anborgh PH, Murdoch B, Bhatia M, Nakanishi S, et al. G protein-coupled receptor kinase-mediated desensitization of metabotropic glutamate receptor 1A protects against cell death. J Biol Chem. 2000; 275:38213–20. [PubMed: 10982802]

De Sarno P, Shestopal SA, King TD, Zmijewska A, Song L, Jope RS. Muscarinic receptor activation protects cells from apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition. J Biol Chem. 2003; 278:11086–93. [PubMed: 12538580]

De Sarno P, Shestopal SA, Zmijewska AA, Jope RS. Anti-apoptotic effects of muscarinic receptor activation are mediated by Rho kinase. Brain Res. 2005; 1041:112–5. [PubMed: 15804506]

DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, Bunnett NW. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. Proc Natl Acad Sci U S A. 2000; 97:11086–91. [PubMed: 10995467]

Europe PMC Funders Author Manuscripts

Diao CT, Li L, Lau SY, Wong TM, Wong NS. kappa-Opioid receptor potentiates apoptosis via a phospholipase C pathway in the CNE2 human epithelial tumor cell line. Biochim Biophys Acta. 2000; 1499:49–62. [PubMed: 11118638]

Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. Nat Rev Cancer. 2007; 7:79–94. [PubMed: 17251915]

Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet. 2003; 33:357–65. [PubMed: 12567188]

Erster S, Mihara M, Kim RH, Petrenko O, Moll UM. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol Cell Biol. 2004; 24:6728–41. [PubMed: 15254240]

Fang X, Yu S, LaPushin R, Lu Y, Furui T, Penn LZ, et al. Lysophosphatidic acid prevents apoptosis in fibroblasts via G(i)-protein-mediated activation of mitogen-activated protein kinase. Biochem J. 2000; 352(Pt 1):135–43. [PubMed: 11062066]

Haupt S, Haupt Y. Importance of p53 for cancer onset and therapy. Anticancer Drugs. 2006; 17:725–32. [PubMed: 16926623]

Hu T, Miller CM, Ridder GM, Aardema MJ. Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL, and CHL: implications for genotoxicity testing. Mutat Res. 1999; 426:51–62. [PubMed: 10320750]

Kakudo Y, Shibata H, Otsuka K, Kato S, Ishioka C. Lack of correlation between p53-dependent transcriptional activity and the ability to induce apoptosis among 179 mutant p53s. Cancer Res. 2005; 65:2108–14. [PubMed: 15781620]

Klabunde T, Hessler G. Drug design strategies for targeting G-protein-coupled receptors. Chembiochem. 2002; 3:928–44. [PubMed: 12362358]

Koh JY, Palmer E, Cotman CW. Activation of the metabotropic glutamate receptor attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. Proc Natl Acad Sci U S A. 1991; 88:9431–5. [PubMed: 1658782]

Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science. 1999; 285:1733–7. [PubMed: 10481009]

Kwon YG, Min JK, Kim KM, Lee DJ, Billiar TR, Kim YM. Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. J Biol Chem. 2001; 276:10627–33. [PubMed: 11134047]

Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R, Brady JN. Phosphorylation of p53 serine 15 increases interaction with CBP. J Biol Chem. 1998; 273:33048–53. [PubMed: 9830059]

Li DW, Liu JP, Schmid PC, Schlosser R, Feng H, Liu WB, et al. Protein serine/threonine phosphatase-1 dephosphorylates p53 at Ser-15 and Ser-37 to modulate its transcriptional and apoptotic activities. Oncogene. 2006; 25:3006–22. [PubMed: 16501611]

Marchenko ND, Zaika A, Moll UM. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem. 2000; 275:16202–12. [PubMed: 10821866]

Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci. 2001; 22:368–76. [PubMed: 11431032]

Meek DW. Mechanisms of switching on p53: a role for covalent modification? Oncogene. 1999; 18:7666–75. [PubMed: 10618706]

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, et al. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. 1994; 9:1799–805. [PubMed: 8183579]

Moll UM, Wolff S, Speidel D, Deppert W. Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol. 2005; 17:631–6. [PubMed: 16226451]

Murga C, Laguinge L, Wetzker R, Cuadrado A, Gutkind JS. Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. J Biol Chem. 1998; 273:19080–5. [PubMed: 9668091]

Murph MM, Hurst-Kennedy J, Newton V, Brindley DN, Radhakrishna H. Lysophosphatidic acid decreases the nuclear localization and cellular abundance of the p53 tumor suppressor in A549 lung carcinoma cells. Mol Cancer Res. 2007; 5:1201–11. [PubMed: 18025263]

Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell. 2001; 7:683–94. [PubMed: 11463392]

Nemajerova A, Erster S, Moll UM. The post-translational phosphorylation and acetylation modification profile is not the determining factor in targeting endogenous stress-induced p53 to mitochondria. Cell Death Differ. 2005; 12:197–200. [PubMed: 15550936]

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science. 2000; 288:1053–8. [PubMed: 10807576]

Park BS, Song YS, Yee SB, Lee BG, Seo SY, Park YC, et al. Phospho-ser 15-p53 translocates into mitochondria and interacts with Bcl-2 and Bcl-xL in eugenol-induced apoptosis. Apoptosis. 2005; 10:193–200. [PubMed: 15711935]

Paulsen MT, Starks AM, Derheimer FA, Hanasoge S, Li L, Dixon JE, et al. The p53-targeting human phosphatase hCdc14A interacts with the Cdk1/cyclin B complex and is differentially expressed in human cancers. Mol Cancer. 2006; 5:25. [PubMed: 16784539]

Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 1998; 12:2831–41. [PubMed: 9744860]

Sayan BS, Sayan AE, Knight RA, Melino G, Cohen GM. p53 is cleaved by caspases generating fragments localizing to mitochondria. J Biol Chem. 2006; 281:13566–73. [PubMed: 16531411]

Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell. 1997; 91:325–34. [PubMed: 9363941]

Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev. 1997; 11:3471–81. [PubMed: 9407038]

Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, et al. M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res. 2007; 67:3936–44. [PubMed: 17440109]

Strom E, Sathe S, Komarov PG, Chernova OB, Pavlovska I, Shyshynova I, et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. Nat Chem Biol. 2006; 2:474–9. [PubMed: 16862141]

Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, et al. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev. 1999; 13:152–7. [PubMed: 9925639]

Tobin AB, Budd DC. The anti-apoptotic response of the Gq/11-coupled muscarinic receptor family. Biochem Soc Trans. 2003; 31:1182–5. [PubMed: 14641022]

Ullrich SJ, Mercer WE, Appella E. Human wild-type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells. Oncogene. 1992; 7:1635–43. [PubMed: 1630823]

Unger T, Juven-Gershon T, Moallem E, Berger M, Vogt Sionov R, Lozano G, et al. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. Embo J. 1999; 18:1805–14. [PubMed: 10202144]

van Koppen CJ, Kaiser B. Regulation of muscarinic acetylcholine receptor signaling. Pharmacol Ther. 2003; 98:197–220. [PubMed: 12725869]

Vichalkovski A, Baltensperger K, Thomann D, Porzig H. Two different pathways link G-protein-coupled receptors with tyrosine kinases for the modulation of growth and survival in human hematopoietic progenitor cells. Cell Signal. 2005; 17:447–59. [PubMed: 15601623]

Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000; 408:307–10. [PubMed: 11099028]

Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer. 2002; 2:594–604. [PubMed: 12154352]

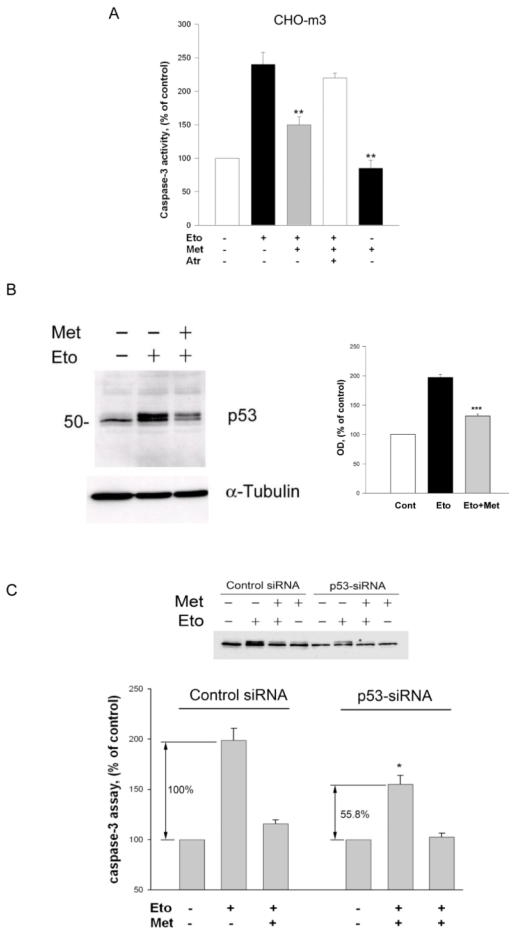
Yan GM, Lin SZ, Irwin RP, Paul SM. Activation of muscarinic cholinergic receptors blocks apoptosis of cultured cerebellar granule neurons. Mol Pharmacol. 1995; 47:248–57. [PubMed: 7870032]

Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. Proc Natl Acad Sci U S A. 1999; 96:14517–22. [PubMed: 10588737]

Zhou Y, Larsen PH, Hao C, Yong VW. CXCR4 Is a Major Chemokine Receptor on Glioma Cells and Mediates Their Survival. J. Biol. Chem. 2002; 277:49481–49487. [PubMed: 12388552]

Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. Proc Natl Acad Sci U S A. 2001; 98:1607–12. [PubMed: 11171998]

Europe PMC Funders Author Manuscripts



Europe PMC Funders Author Manuscripts

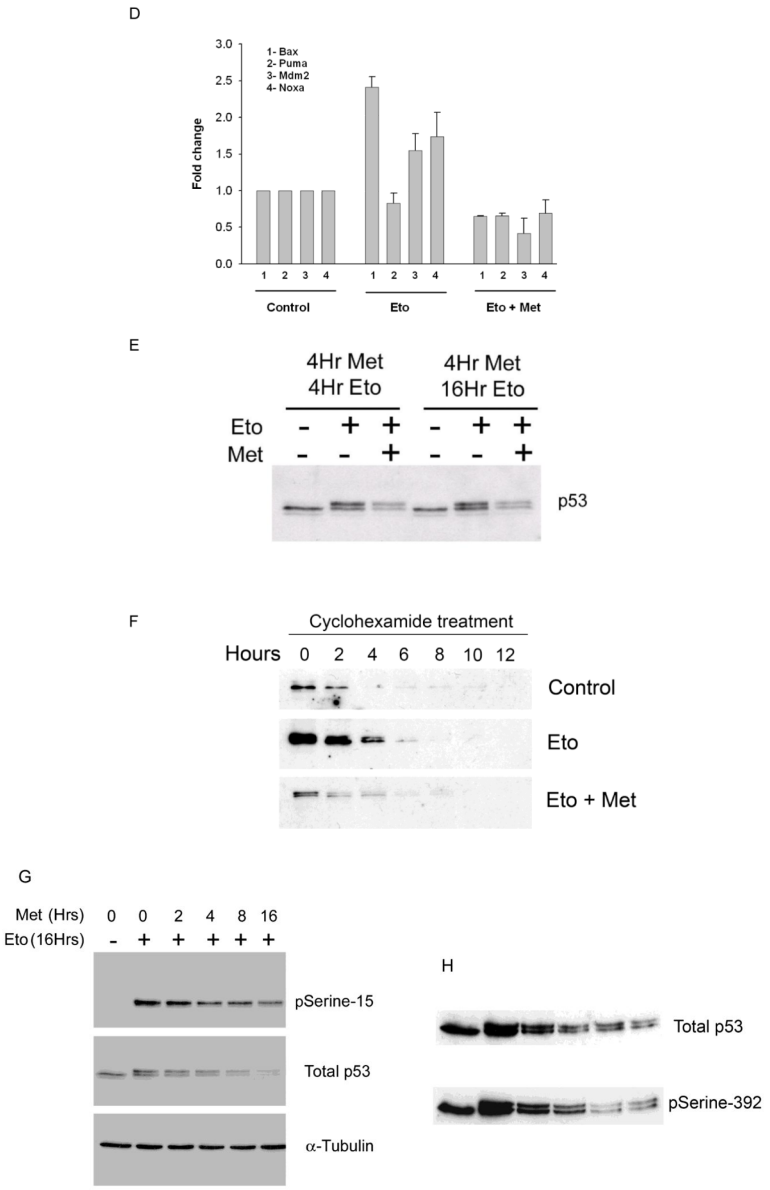


Figure 1. M_3-muscarinic receptors protect CHO-m3 cells from etoposide-induced apoptosis and attenuated the up-regulation of p53 expression

CHO cells stably expressing the human M_3-muscarinic (CHO-m3) receptor were exposed to etoposide (250μM; Eto) for 16hrs in the absence or presence of methycholine (1mM; Met) and atropine (Atr; 10μM). Atropine was added at the same time as methylcholine. The cells were then lysed and either (A) caspase 3 activity determined as described in materials and methods or (B) the lysate (20μg protein) was Western blotted for p53 expression with α-tubulin probed as a loading control. Shown in the right hand panel is quantification of p53 expression levels. (C) CHO-m3 cells were transfected with either control or p53 specific siRNA duplexes (50pmols). 48 hours following transfection cells were treated with or without etoposide (250μM; Eto) in the presence or absence of methacholine (1mM; Met) for 16 hours after which cell lysates were prepared and either processed for caspase 3 activity or probed in Western blots for p53 expression. (D) Gene expression changes measured by RT-PCR, of Bax, Puma, Mdm2 and Noxa genes following etoposide (250μM; Eto) treatment in the absence and presence of methacholine (1mM; Met). (E) CHO-m3 cells were treated with

etoposide (250μM; Met) for 4 or 16 hours. Methylcholine (1mM; Met) was applied simultaneously with etoposide. The action of methylcholine was stopped after four hours by the addition of atropine (10μM). Etoposide treatment was either stopped at the point of atropine addition (i.e. 4 hours) or allowed to continue for a total of 16 hours. Cell lysates were then prepared and p53 levels determined by Western blot. (F) Following etoposide (250μM; Eto) treatment in the absence or presence of methacholine (1mM; Met) for 16 hours protein synthesis was inhibited by the addition of cyclohexamide (2μg/ml) and the incubation continued for the times indicated. The incubations were stopped by preparation of a cell lysate that was then probed for p53 expression in Western blots.. (G and H) CHO-m3 cells were stimulated in the presence and absence of etoposide (250mM; Eto) with or without methacholine (1mM; Met). At the times indicated stimulation with methylcholine was stopped by the addition of atropine (10μM) and the incubation with etoposide continued for a total of 16 hours. Cells were then lysed and lysates probed in Western blots for p53 and serine-15 phosphorylation (G) or p53 and serine-392 phosphorylation (H).

Western blots shown are typical of at least three experiments. The graphical results represent the mean (± SE) of 6 independent experiments. *** $p<0.001$,**$p<0.01$; *$p<0.05$, paired Students *t*-test; represents significant difference from etoposide only treatment except for figure 1C where * represents significant difference from etoposide treatment in the presence of control siRNA..

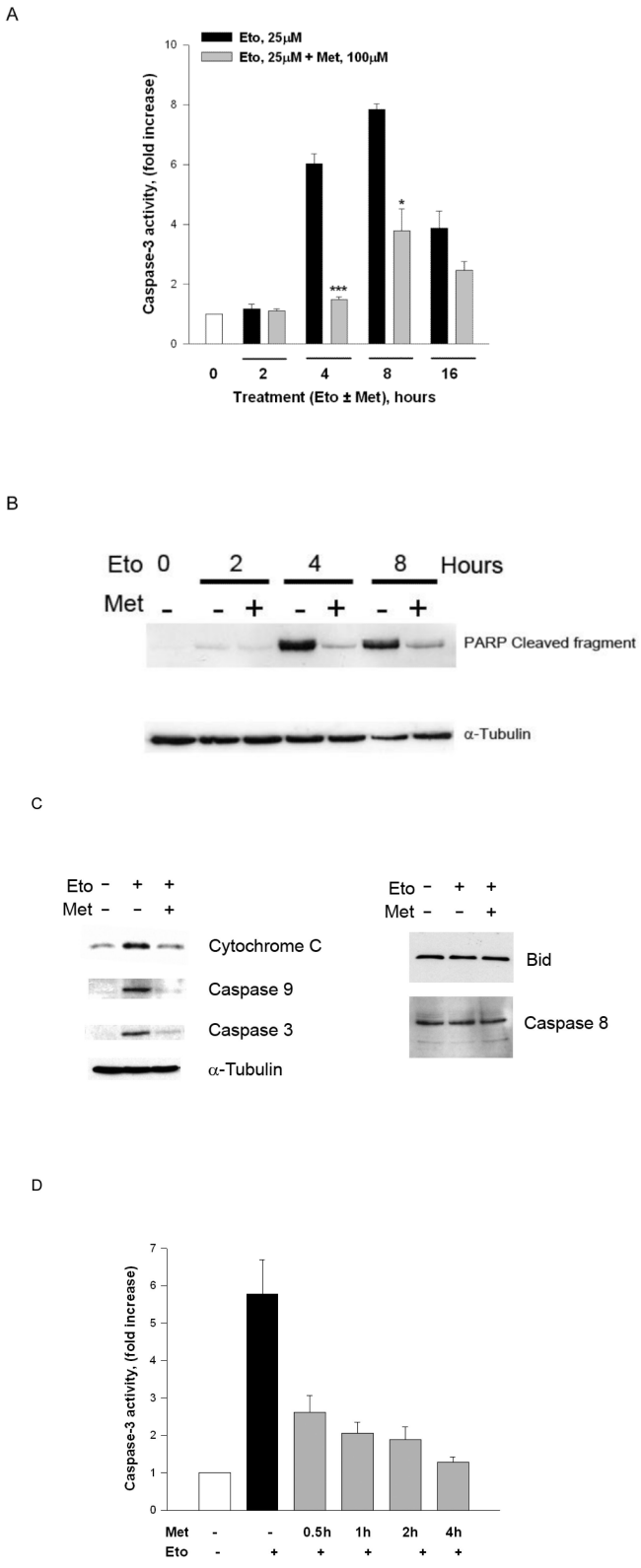


Figure 2. The M_3-muscarinic receptor protects SH-SY5Y cells from DNA-damage induced apoptosis

SH-SY5Y cells were treated with etoposide (Eto, 25μM) in the presence or absence of methylcholine (Met, 100μM) for 2-16 hours. After treatment, cells were lysed and lysates processed either for (A) caspase-3 activity, (B) Western blotting for PARP cleavage or (C) Western blotted for cytsolic cytochrome C, cleaved caspase 9 and cleaved caspase 3 together with Western blots for Bid and caspase 8. (D) SH-SY5Y cells were treated with etoposide (Eto, 25μM) in the presence or absence of methylcholine (Met, 100μM) for 4 hours. During this period methylcholine stimulation was stopped by addition of the antagonist atropine (10μM) at the indicated times. Lysates were prepared at the end of the experiment and caspase activity determined.

The Western blots shown are typical of at least three experiments. The graphical results represent the mean (± SE) of at least 3 independent experiments. *** $p<0.001$, * $p<0.05$; paired Students *t*-test; represents significant difference from etoposide only treatment.

Europe PMC Funders Author Manuscripts

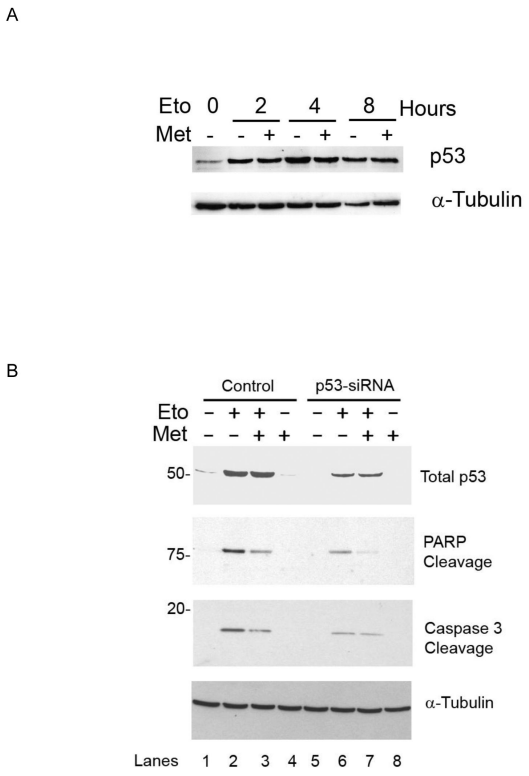


Figure 3. p53-mediates the apoptotic response to etoposide in SH-SY5Y but M_3-muscarinic receptors protect against apoptosis independently of changes in p53 expression

(A) SH-SY5Y cells treated with etoposide (Eto, 25μM) in the absence or presence of methylcholine (Met, 100μM) were lysed and the lysates processed for p53 expression. (B) SH-SY5Y cells were transfected with either siRNA duplex targeted to p53 or control duplex. 48 Hours later cells were treated for four hours with etoposide (Eto, 25mM) in the presence or absence of methacholine (Met 100μM). Cell Lysates were then prepared and probed for p53, PARP cleavage and caspase 3 processing. Shown is a typical experiment of at least three independent experiments.

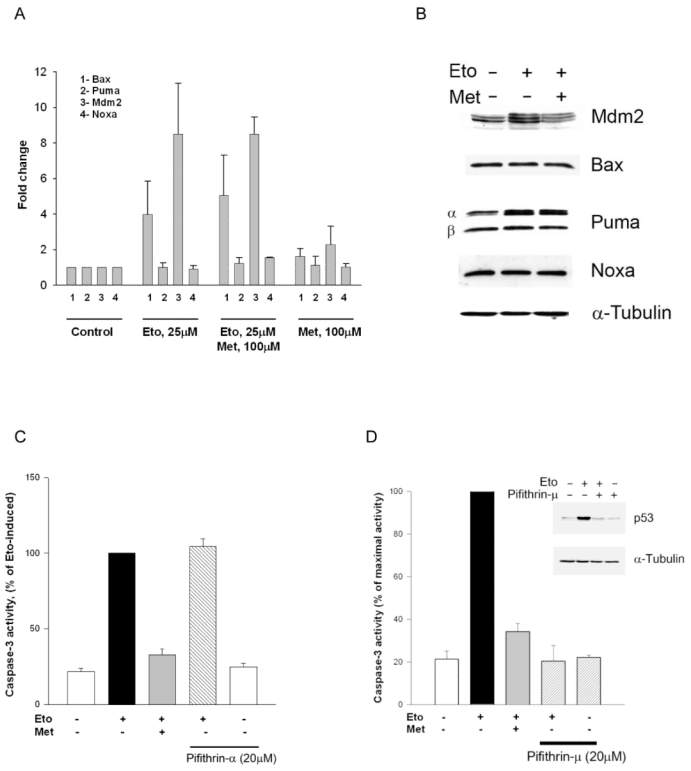


Figure 4. M_3-muscarinic receptors protect against apoptosis in SH-SY5Y cells independently p53-mediated transactivation

(A) Gene expression changes measured by RT-PCR, of Bax, Puma, Mdm2 and Noxa genes in SHSY-5Y cells treated for four hours with etoposide (Eto, 25μM) in the absence or presence of methylcholine (Met, 100μM). (B) Cells treated with etoposide and methycholine were lysed and the lysates probed for the expression of p53 responsive gene products Mdm2, Bax, Puma and Noxa. (C) Where indicated, pifithrin-α (20μM), an inhibitor for p53 transactivation, or (D) pifithrin-μ (20μM), an inhibitor of p53, were added to cells 30 minutes before etoposide and methylcholine and maintained for the duration of the experiment (4 hours). Cells were then lysed and lysates used in caspase assays. The Western blots shown were typical of at least three independent experiments. Graphical results represent the mean (± SE) of 3 independent experiments.

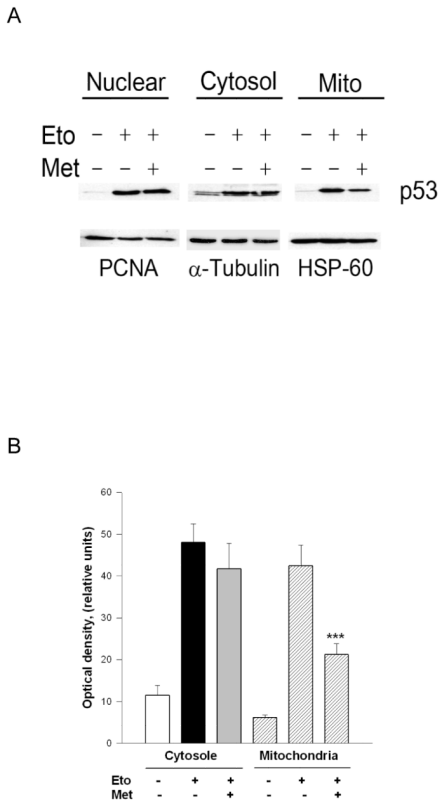


Figure 5. M_3-muscarinic receptors regulate p53 translocation to the mitochondria

SH-SY5Y cells were treated for 4 hours with etoposide (Eto, 25μM) in the presence or absence of methylcholine (Met, 100μM). Following treatment, cells were placed on ice and immediately used for fractionation into nuclear, cytosolic and mitochondrial fractions (see; materials and methods). Isolated fractions were used for western blotting using; (A) anti-p53 antibodies. Antibodies against PCNA, α-tubulin and HSP-60 were used as markers for nuclear, cytosolic and mitochondrial fractions, respectively. (B) Quantification of the data shown in (A). Western blots shown are representative of at least three independent experiments. Graphical results represent the mean (± SE) of the experiments illustrated in the Western blots. *** $p<0.001$; paired Students *t*-test; represents significant difference from etoposide only treatment.

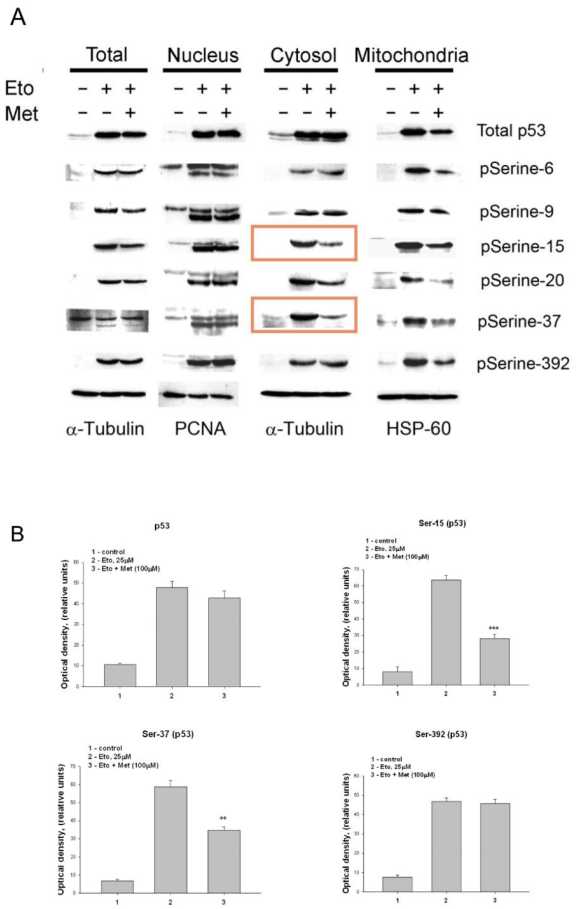


Figure 6. M_3-muscarinic receptors regulate the phosphorylation status of p53 located in the cytoplasm

(A) SH-SY5Y cells were treated for 4 hours with etoposide (Eto, 25μM) in the presence or absence of methylcholine (Met, 100μM). Following treatment, cells were placed on ice and immediately used for fractionation into nuclear, cytosolic and mitochondrial fractions (see; materials and methods). Isolated fractions were used in Western blots and probed for the phosphorylation of p53 at serines 6, 9, 15, 20, 37 and 392. Antibodies against PCNA, α-tubulin and HSP-60 were used as markers for nuclear, cytosolic and mitochondrial fractions, respectively. (B) Quantification of the cytosolic p53 and cytosolic p53 phosphorylated at serine-15, 37 and 392 – from data shown in (A). Western blots shown are representative of at least three independent experiments. Graphical results represent the mean (± SE) of the experiments illustrated in the Western blots. ** $p<0.01$, *** $p<0.001$; paired Students *t*-test; represents significant difference from etoposide only treatment. Orange boxes highlight the results for phosphorylation of cytoplasmic p53 on serine-15 and serine-37.