PHARMACOLOGY OF TAURINE

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Today I would like to discuss the regulation of apoptosis by angiotensin II and the amino acid, taurine. The amino acid, taurine, is found in very high concentrations in most tissues, although it appears to be highest in excitable tissues. When the intracellular concentration of taurine is dramatically decreased, several defects begin to develop. These include a retinopathy, a cardiomyopathy and immune deficiency. The basic mechanism underlying these defects is poorly understood.

FIGURE 1

It has been established by Novotny and coworkers, that the cardiomyopathy is characterized by abnormalities in both systolic and diastolic function. In the taurine deficient cat, the initial abnormality to develop is diastolic dysfunction. This defect is illustrated in the First slide taken from the work of Novotny et al. showing a P-V relationship for the taurine deficient and normal heart. You can readily see that the taurine deficient heart is shifted to the right, indicating that the heart is more complaint. In other words, it tends to be flabby. Echocardiographic studies by the same group reveal an increase in chamber volume, leading Novotny and coworkers to suggest that taurine deficiency causes an eccentric form of hypertrophy.

FIGURE 2

This form of hypertrophy is associated with an increase in both the cross-section area and the length of the myocyte. Thus, taurine deficiency affects the cell's ability to synthesize protein.

It is widely accepted among cardiac researchers that two important factors contribute to the development of hypertrophy in the heart. One of the mechanisms involves cell stretching. When cells are stretched, several signaling pathways are initiated within cells that culminate in the activation of hypertrophic effectors, such as the MAP kinases, the early response genes and the JAK/Stat pathway. The other mechanism linked to the development of hypertrophy is the upregulation of neurohumoral agents. One of the most important of these agents is angiotensin II. It has been shown that over-expression of angiotensin II type I receptor induces cardiac hypertrophy and remodeling. Moreover, reducing either the production of renin, the conversion of angiotensin I to angiotensin II or the activation of the angiotensin II receptor prevents the development of hypertrophy. In this regard it is important that the ACE and angiotensin II receptor inhibitors are mainstay therapy in congestive heart failure.

In addition to promoting hypertrophy, angiotensin II also induces apoptosis. Although there is considerable debate regarding the contribution of apoptosis to the development of overt heart failure, there is no dispute that apoptosis is observed in the failing heart. In fact, in one study the number of apoptotic cells in the failing heart approached 35.5%. According to some investigators the extent of apoptosis is directly correlated with the severity of heart failure. Moreover, the transition from compensated hypertrophy to overt heart failure is associated with the loss of cardiomyocytes, as apoptotic genes become upregulated. Thus, it is logical to assume that angiotensin II-induced apoptosis might contribute to ventricular remodeling and onset of overt heart failure.

Despite the potential importance of angiotensin II-induced apoptosis to the failing heart, the mechanism underlying the development of apoptosis is poorly understood. Anversa and coworkers have suggested that the activation of calcium-dependent nucleases is important in development of angiotensin

II. However, the cleavage of DNA occurs in the latter stages of apoptosis. We favor the hypothesis advance Griendling and coworkers who have shown that angiotensin II activated NADPH oxidase, leading to the generation of reactive oxygen species.

FIGURE 3

They went on to propose that reactive oxygen species activate JNK, which in turn promotes apoptosis. However, this scheme appears to exclude several important steps. Therefore, our initial goal was to identify some of these missing steps. Since oxidative DNA damage is common, we first explored the possibility that angiotensin II might cause DNA damage.

The technique used to examine DNA damage utilized Southern blot analysis. DNA was isolated from myocytes exposed to medium containing or lacking 1nM angiotensin II.

FIGURE 4

The DNA was digested overnight with the restriction enzyme, Bam HII. The samples were then exposed to 0.1 N NaOH, which had a two-fold effect. First, the NaOH cleaved the DNA at sites of base modification. Second, it weakened the interaction between the two strands of DNA, allowing separate migration during electrophoresis. After a transfer and an autoradiography step, the intensity of the prominent 10 kbase DNA band was determined. The frequency of stand breaks was calculated from the intensity of the high molecular weight band.

The next slide reveals that isolated cardiomyocytes exposed to 1 nM angiotensin II experience significant mitochondrial DNA damage.

FIGURE 5

While control cells contained only high molecular weight DNA, cells exposed to 1 nM angiotensin II exhibited reduced levels of the 10 kbase strand. Although not shown on this slide, the angiotensin II treated cells contained significant levels of lower molecular weight species, clear evidence of strand breaks. However, after 24 hours of incubation, much of the DNA damage had been repaired.

Angiotensin II-induced DNA damage was prevented by inclusion of the NADPH oxidase inhibitor, diphenylene iodonium (DPI), in the incubation medium.

FIGURE 6

This effect was seen at all three time-points. Note that DPI had no effect on the control cells.

These data suggested that superoxide plays a central role in angiotensin II-mediated DNA damage. However, it has also been reported in the literature that angiotensin II promotes the upregulation of the inducible form of nitric oxide synthase. Therefore, nitric oxide or the combination of nitric oxide and superoxide, namely peroxynitrite, might cause the DNA damage. To examine the role of iNOS in angiotensin II-mediated DNA damage, the cells were incubated with medium containing both angiotensin II and the iNOS inhibitor, aminoguanidine.

FIGURE 7

As seen on this slide, cells treated with angiotensin II and aminoguanidine exhibited no DNA damage.

Work in our department had shown that nitric oxide modifies DNA by causing the deamination of purines, whereas superoxide caused extensive modification of thymine and guanine. To identify the type of nucleotide damage, as well as the pattern of angiotensin II-meidated DNA damage, LM-PCR was performed on a 200 bp stretch to mitochondrial DNA isolated from cells treated with angiotensin II. The pattern of damage in these cells was compared with cell treated with nitric oxide, superoxide or peroxynitrite. The pattern of damage is shown on the next slide.

FIGURE 8

Some of the DNA was treated with 0.1 N NaOH to cleave DNA at the site of basic modification. Others were subjected to LM-PCR without NaOH treatment. The sequence of bases in the region examined is shown on the far left. Note the sequence of 3 guanines. These 3 bases are modified by xanthine oxidase/xanthine (a superoxide generator). However, only two of the 3 guanines are modified by nitric oxide, peroxynitrite or angiotensin II. We replotted the data using the Maxim-Gilbert sequencing ladder, as seen on the next slide

FIGURE 9

The damage is represented by the bars, with the height of each bar indicating the degree of base damage. Note that superoxide caused the greatest damage and nitric oxide the least. Particularly obvious from this slide is that the pattern of damage is nearly identical for peroxynitrite and angiotensin II. Therefore, we concluded that peroxynitrite is the oxidizing agent responsible for angiotensin II-mediated DNA damage.

DNA damage is known to activate the suppressor gene, p53, through a series of phosphorylation reactions.

FIGURE 10

In agreement with its effects on DNA damage, it was found that angiotensin II enhanced the phosphorylation of p53 at one of its phosphorylation sites, serine 20. This phosphorylation reaction is important, not only because it enhances the activity of p53, but also because it prevents its turnover. Phosphorylation of p53 at another serine residue, serine 15, was also promoted by DNA damage. Unfortunately, DPI interfered with the phosphorylation of both serine 15 and serine 20, however, we were able to detect an effect of aminoguanidine on the phosphorylation of serine 15.

FIGURE 11

While angiotensin II stimulated the phosphorylation of serine 15 by 75%, aminoguanidine reduced the enhancement of phosphorylation to about 30%. Therefore, we were able to conclude that iNOS contributed to the activation of p53, although the effect of NADPH oxidase was inconclusive.

One of the important functions of p53 is to stop replicative DNA synthesis, allowing time for the cell to repair any DNA damage. If a cell is unable to repair the DNA damage, p53 promotes the cell's death via apoptosis. In a previous study, Offer et al showed that the level of accumulated DNA damage determines whether p53 promotes DNA repair or induces apoptosis. One way the cell recognized the degree of DNA damage is the phosphorylation pattern of p53. We have not fully explored the phosphorylation status of p53 form the angiotensin II-treated cells. Nonetheless, we do know that approximately 1 base/10 kbases are modified by angiotensis II. This amount of DNA damage appears to be sufficient to activate the apoptotic cascade.

P53 can function as a transcriptional activator and as a transcriptional repressor.

FIGURE 12

Both properties have been implicated in p53-mediated apoptosis. One of the targets of transcriptional activation is the pro-apoptotic factor, Bax. Bax is a member of the Bcl-2 family of proteins and is capable of interacting with mitochondria, causing the release of cytochrome c. As we will discuss later, cytochrome c is a key step in the initiation of apoptosis. Because of its role as a key regulator of apoptosis, the content of Bax was determined in cells prior to and following exposure to medium containing angiotensin II. As seen in the slide, angiotensin II elevated Bax content. This upregulation of Bax was blocked by DPI, indicating that the activation of NADPH oxidase was responsible for the increase in Bax content.

Although the increase in Bax content was highly significant, the effectiveness of Bax in promoting apoptosis also depends upon the cellular levels of the anti-apoptotic Bcl-2 family members.

FIGURE 13

These proteins, of which Bcl-2 is a prototypic member, are capable of interacting with Bax, thereby neutralizing its activity. Moreover, Bcl-2 interacts with the mitochondria, preventing cytochrome c relase. Since p53 downregulates Bcl-2 in breast cancer cells, it was logical to assume that angiotensin II might also reduce Bcl-2 levels in the myocyte. The effect of angiotensin II on Bcl-2 was time-dependent, with levels falling within 1 hr and rebounding thereafter.

The effect of DPI on angiotensin-mediated alterations in Bax and Bcl-2 are shown on the next slide.

FIGURE 14

After 6 hr of angiotensin II exposure, Bcl-2 levels fell about 15% and Bax levels were increased about 25%. The Bax/Bcl-2 ratio was elevated about 40%. DPI had no effect on the ratio in the absence of angiotensin II, but it completely blocked the elevation in the ratio mediated by angiotensin II.

The effects of Bax and Bcl-2 are mediated by the mitochondria.

FIGURE 15

Bax can interact with the mitochondria to open pores that lead to the release of cytochrome c. In the cytosol, cytochrome c interacts with the protein, apaf-1, which in the presence of dATP can activate one of the initiating caspases, caspase 9. The initiating caspases are proteolytic enzymes that activate effector caspases, such as caspases 3, 6, and 7, which in turn cleave important proteins, causing apoptotic cell death. Bcl-2 and Bax regulate this process by altering the release of cytochrome c from the mitochondria.

If angiotensin II acts through this mitochondrial mechanism, it should cause the activation of caspase 9.

FIGURE 16

However, there are two other mechanisms that could consequently account for angiotensin II-induced apoptosis. One is a receptor mechanism, which leads to the activation of caspases 2 and 8. The other is an endoplasmic reticular mechanism that leads to the activation of caspase 12.

This slide shows that in the angiotensin II treated cell, pro-caspase 9 is converted to caspase 9.

FIGURE 17

Western blot analysis revealed that the content of two cleavage products (the 40 and 38 kD bands) were elevated in angiotensin II treated cells. Pro-caspase 9 is the 50 kD band. When the data were expressed as the active caspase 9/pro-caspase 9 ratio, it was concluded that angiotensin II caused a 25% increase in the conversion of pro-caspase 9 to caspase 9. This observation is consistent with the TUNEL assay, which showed that angiotensin II caused 12% of the cells in culture to undergo apoptosis.

These data allow us to modify the original scheme of Griendling, as shown on the next slide.

FIGURE 18

We now suggest that angiotensin II activates NADPH oxidase, which is responsible for the generation of free radicals. These reactive oxygen species cause DNA damage, leading to the activation of p53. Through transcriptional activation, Bax is upregulated. Bax in turn acts through the mitochondiral mechanism to initiate apoptosis.

I would like to now discuss the potential importance of this mechanism as it relates to taurine deficient heart failure. It has been shown that cats made deficient of the amino acid, taurine, develop a cardiomyopathy. It is our feeling that this cardiomyopathy is caused by the potentiation of angiotensin II action. Since angiotensin II induces apoptosis and heart failure has been linked to apoptosis, we proposed that taurine deficiency must potentiate angiotensin II-induced apoptosis. To study this hypothesis, taurine deficiency was produced by incubating isolated cardiomyocytes with medium containing the taurine transport inhibitor, B-alanine. After three days, the cellular content of taurine was decreased by 50%. The cells were than incubated for either 12 or 24 hours with medium containing 1 nM angiotensin II. Two measures of apoptosis were evaluated: TUNEL staining and caspase 9 activation. We found that about 12% of the control myocytes exhibited the characteristic brown staining pattern of TUNEL positive cells.

FIGURE 19

Exposure of the cells to angiotensin II increased the number of apoptotic cells to 24%. B-alanine treatment did not increase the number of TUNEL positive cells in the absence of angiotensin II. However, 24 hours after exposure to angiotensin II, 35% of the cells were TUNEL positive.

The next two slides show that the activation of caspase 9 was more complete in the B-alanine treated cells exposed to angiotensin II.

FIGURE 20 & 21

While the caspase 9/pro-caspase 9 ratio was significantly increased in normal cells treated with angiotensin II, the extent of activation was enhanced in the taurine deficient cells exposed to angiotensin II. These data clearly show that taurine deficiency potentiates angiotensin II-induced apoptosis.
The question then becomes - How can taurine affect angiotensin II-induced apoptosis?

FIGURE 22

The next slide shows the chemical structure of taurine. It is a simple compound, with an amino group and a sulfonic acid moiety. Based on its structure, it is obvious that taurine cannot be a scavenger of most reactive oxygen species. An exception is HOCl, which reacts with the amino group of taurine to form taurine chloramine. Although taurine is not a direct scavenger of free radicals, the literature is full of examples in which taurine limits either oxidative stress of the consequences of oxidative stress. Therefore, taurine affects some other event in the signaling pathway of angiotensin II.

One possibility is that taurine affects the extent of angiotensin II-induced DNA damage. This possibility was addressed by analyzing DNA damage using Southern blot analysis.

FIGURE 23

Exposure of cells to medium containing 20 mM taurine had no effect on the frequency of strand breaks observed 1 hour and 3 hours following exposure to 1 nM angiotensin II. However, taurine treatment did affect the extent of DNA damage seen after 24 hours of angiotensin II exposure. At the latter time point, DNA repair reduced the frequency of strand breaks form 1/10kbases to0.4/10 kbases in untreated cells exposed to angiotensin II. The frequency of strand breaks was reduces further to 0.1/10 kbases in the taurine treated cells. Although these data support the notion that taurine affects DNA repair, the effect is not large and unlikely to be the primary site of taurine action.

Taurine also reduces the degree of p53 activation.

FIGURE 24

While there was a significant increase in the degree of p53 phosphorylation at serine 20 in untreated cells exposed to angiotensin II, this effect was dramatically attenuated in cells pretreated with 20 mM taurine prior to exposure to 1 nM angiotensin II.

P53 is capable of inducing apoptosis through several mechanisms. As discussed earlier, one of these mechanisms involves the upregulation of Bax.

FIGURE 25 & 26

We found that while angiotensin II treatment elevated Bax content in the untreated cell by 60%, Bax levels rose more than 2 fold in the B-alanine treated cell exposed to angiotensin II. Nonetheless, this impressive effect was somewhat muted by an upregulation of Bcl-2 in the taurine deficient cell. As a result of the increase in Bcl-2 content, the Bax/Bcl-2 ratio increased 55% in the B-alanine treated cell exposed to angiotensin II rather than the 2-fold increase that would have occurred without the elevation in Bcl-2 levels.

FIGURE 27

Release of cytochrome c from the mitochondria can involve either Bax-linked pores or the mitochondrial permeability transition pore. The permeability transition pore consists of at least three components, voltage dependent anion channels (VDAC), adenine nucleotide translocase (ANT) and cyclophilin D. When the three are coupled to form the pore, a collapse of the membrane potential, mitochondrial swelling and rupture of the outer mitochondrial membrane ensues. This pore is regulated by a number of factors, including protein kinase C-epsilon.

Therefore, we examined the effect of taurine and angiotensin II on protein kinase C-epsilon content.

FIGURE 28

Angiotensin II elevated the membrane content of protein kinase C-epsilon by 25% in the normal cells and by 50% in the B-alanine treated cells. The angiotensin II-mediated translocation of protein kinase C-epsilon was also promoted by taurine deficiency. Because this effect would be considered cytoprotective, it would not contribute to the taurine deficient cardiomyopathy.

In summary, angiotensin II appears to induce apoptosis through a mechanism involving the activation of NADPH oxidase.

FIGURE 29

We propose that the generation of reactive oxygen species leads to DNA damage. As a result, p53 is activated. Although p53 can induce apoptosis through several mechanisms, the upregulation of Bax appears to contribute to angiotensin II-induced apoptosis. Finally, taurine inhibits angiotensin II-induced apoptosis by interfering with the activation of p53.