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Failure to Protect the Myocardium Against Ischemia/Reperfusion Injury After Chronic Atorvastatin Treatment Is Recaptured by Acute Atorvastatin Treatment

A Potential Role for Phosphatase and Tensin Homolog Deleted on Chromosome Ten?

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OBJECTIVES We sought to ascertain whether chronic oral therapy with atorvastatin protects against ischemia/reperfusion (I/R) injury.

BACKGROUND We have recently shown that acute atorvastatin treatment protects against reperfusion-induced injury by activating the PI3K/Akt/eNOS pathway. However, many patients are on chronic statin therapy, and it is necessary to investigate whether this, in itself, provides a therapeutic advantage.

METHODS Sprague-Dawley rats were orally treated for one day, three days, one week, or two weeks with 20 mg/kg of atorvastatin or vehicle, after which the hearts underwent 35 min of ischemia and 120 min reperfusion (IR). Two additional groups were treated for one or two weeks with atorvastatin and then received a supplementary dose of 40 mg/kg before IR. The risk zone was determined using Evans blue and infarct size (IR%) using triphenyltetrazolium chloride staining.

RESULTS Treatment with atorvastatin for one and three days significantly reduced infarct size versus controls (38.9 ± 3.1% vs. 56.4 ± 2.3%; 39.3 ± 2.4% vs. 61.3 ± 3.8%, respectively). However, after one or two weeks of treatment, no protection was observed (52.6 ± 3.8% vs. 58.6 ± 4.3%; 58.3 ± 2.7% vs. 52.4 ± 5.7%, respectively). Surprisingly, a supplementary dose of atorvastatin recaptured the protection in the groups treated chronically (36.2 ± 2.8% vs. 58.6 ± 4.3%; 26.8 ± 1.5% vs. 51.2 ± 6.7%, at one and two weeks, respectively). Interestingly, we observed an increased level of phosphatase and tensin homolog deleted on chromosome ten (PTEN), the phosphatidylinositol-3 kinase inhibitor, in the chronic treated hearts.

CONCLUSIONS In conclusion, atorvastatin appears to have an acute protective effect that wanes with time associated with an increase in PTEN levels. This waning protection can be recaptured by an acute high dose given immediately before IR. These results may have potential clinical relevance. (J Am Coll Cardiol 2005;45:1287–91) © 2005 by the American College of Cardiology Foundation

The 3-hydroxy-3-methylglutaryl CoA reductase inhibitor drugs, commonly termed statins, are widely used for the treatment of hypercholesterolaemia, a significant risk factor for ischemic heart disease. They act by inhibiting the rate-limiting step in cholesterol synthesis, the conversion of

See page 1292

beta-hydroxy-beta-methylglutaryl-CoA to mevalonate (1). Several studies have shown an improved rate of morbidity and mortality from treatment with statins (2–5). This benefit has been mainly attributed to their cholesterol-lowering properties. However, further analyses of these studies appear to show an extra benefit above and beyond the cholesterol-lowering effects. These other effects have been termed the pleiotropic effects of statins. One such effect is its ability to activate the PI3K)/Akt pro-survival pathway (6), a pathway that, if activated before or after ischemia, has been shown to confer an intrinsic protection from ischemia/reperfusion (I/R) injury (7,8). Statins also have been shown in animal studies to reduce infarct size when given before ischemia (9) or just before reperfusion (10), this effect having been shown to be mediated via PI3K.

However, although it is apparent that such treatment with statins appears to be beneficial when given acutely (10–12), recent studies have shown a detrimental effect after prolonged oral administration, with a reduction in adenosine triphosphate and creatinine phosphate levels in dog myocardium (13,14). It is important to appreciate that although acute activation of the PI3K/Akt pathway is beneficial, promoting cell survival and having an antiapoptotic effect (15–17), chronic activation would cause cardiomegaly (18–20) and could lead to tumor formation, as has been shown in other tissues (21–23). It would therefore

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Abbreviations and Acronyms

eNOS = endothelial nitric oxide synthase
I/R = ischemia/reperfusion
PI3K = phosphatidylinositol-3 kinase
PTEN = phosphatase and tensin homolog deleted on chromosome ten

follow that there should be intrinsic cellular mechanisms able to control PI3K/Akt activation over the course of time. In this regard, phosphatase and tensin homolog deleted on chromosome ten (PTEN) is the phosphatase that reverses the effect of PI3K (24). Therefore, our study examined both acute and chronic oral treatment with atorvastatin on I/R injury. In addition, we measured the level of the phosphatase PTEN, a known inhibitor of the PI3K pro-survival pathway.

METHODS

Atorvastatin treatment. Male Sprague-Dawley rats were used in all experiments. Animal care was performed in accordance with The Guidance on the Operation of the Animals (Scientific Procedure) Act of 1986. Rats weighing between 200 and 250 g (n = 6 per group) were orally treated (gavaged) for either one day, three days, one week, or two weeks with 20 mg/kg of atorvastatin in 1% methylcellulose or methylcellulose alone (control group), after which the hearts were isolated and subjected to I/R on a Langendorff apparatus (as described in the next section). An additional two groups consisted of male Sprague-Dawley rats treated for one week or two weeks with 20 mg/kg atorvastatin, after which they received a further 40 mg/kg of atorvastatin 3 to 4 h before being subjected to myocardial I/R injury on the Langendorff apparatus.

Langendorff perfusion. The rats were anesthetized with 100 mg/kg of pentobarbitone and heparinized with 300 IU intraperitoneally. The hearts were rapidly excised and placed in ice-cold buffer, then mounted on a Langendorff apparatus and retrogradely perfused under constant pressure of 75 mm Hg with modified Krebs-Henseleit buffer, pH 7.35 to 7.45. Myocardial temperature was monitored constantly by a temperature probe inserted in the pulmonary artery outflow track and maintained at 37 ± 0.5°C. Pressure and heart rate were measured using an isovolumetric, fluid-filled latex balloon inserted into the left ventricle via the excised left atrial appendage. Coronary flow also was measured and recorded at regular intervals. A 3-0 surgical suture was placed around the left main coronary artery, a short distance from its origin, and the two ends of the suture were pulled through a small piece of plastic tubing to form a snare.

Experimental protocol for ischemia/reperfusion. The isolated hearts were allowed to stabilize for 30 to 40 min, after which time each heart was subjected to 35 min of regional ischemia, which was induced by tightening the snare around the coronary artery, followed by 120 min of reperfusion, which was induced by releasing the snare.

Infarct size determination. After the reperfusion period, the suture around the coronary artery was re-ligated, and 0.25% Evans blue dye was injected into the heart to delineate the non-risk area. The hearts were then frozen and sliced into 2-mm thick sections and incubated in triphenyltetrazolium chloride for 10 to 15 min at 37°C. The hearts were subsequently fixed in formalin for 24 h and then the risk and infarct areas traced onto acetate sheets. The percentage of infarcted tissue (triphenyltetrazolium chloride negative) developed in the area at risk (Evans blue negative) was calculated using computerized planimetry (Summa Sketch III, Summagraphics, Seymour, Connecticut) and expressed as a percentage of ischemia/reperfusion.

Western blot analyses. SAMPLE COLLECTION. In separate experiments, hearts from Sprague-Dawley rats, gavaged either for one day, three days, one week, or two weeks with 20 mg/kg of atorvastatin in 1% methylcellose or methylcellulose alone, were retrogradely perfused, allowed to stabilize for 20 to 30 min, and then were subjected to 35 min of regional ischemia followed by 5 min of reperfusion (n = 3 per group). At the end of this period, the area at risk was delineated using Evans blue as previously described, excised, freeze clamped, and stored at −80°C for further analysis.

PTEN ANALYSIS. Proteins were extracted by homogenization and centrifugation in cold suspension buffer containing (in mmol/l): NaCl 100, TRIS 10 (pH 7.6), ethylenediamine tetraacetic acid 1 (pH 8.0), sodium pyrophosphate 2, sodium fluoride 2, beta-glycerophosphate 2, phenyl methyl sulfonyl fluoride 0.1 μg/ml, and 1 μg/ml each of aprotinin, leupeptin, trypsin inhibitor, and protease inhibitor. Protein content was determined using a bicinchoninic acid assay (Pierce, Rockford, Illinois). Furthermore, the samples were denaturated by boiling in suspension buffer, which contained (in mmol/l): Tris 100 (pH 6.8), dithiothreitol 200, sodium dodecyl sulfate 2%, bromophenol blue 0.2%, and glycerol 20%. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred on Hybond ECL nitrocellulose membrane (Amersham Biosciences, Amersham, United Kingdom) overnight. Levels of PTEN were detected using primary and secondary antibodies from Cell Signalling Ltd. (United Kingdom) and the enhanced chemiluminescence ECL Western blotting reagent (Amersham Biosciences). The bands were compared using computerized planimetry (NIH Image 1.63).

Statistical analysis. All data were expressed as mean ± standard error of the mean. The data were analyzed using the analysis of variance factorial test. A p value of <0.05 was considered significant.

RESULTS

Infarct/risk ratio. Oral treatment with atorvastatin for one or three days significantly reduced infarct size compared with controls (38.9 ± 3.1% vs. 56.4 ± 2.3% and 39.3 ±

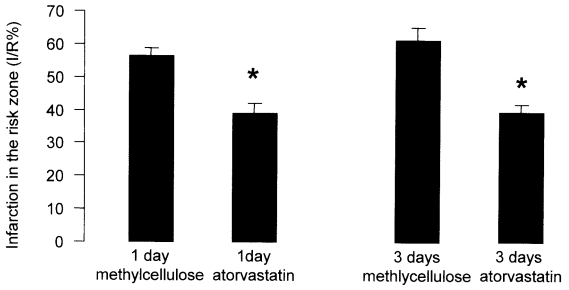

Figure 1. Infarction developed in the risk zone in hearts treated for one or three days with atorvastatin or methylcellulose. *p < 0.05. I/R = ischemia/reperfusion.

2.4% vs. 61.3 ± 3.8%, respectively) (Fig. 1). However, the data shown in Figure 2 demonstrates that, after one or two weeks of oral treatment, the protection was lost (52.6 ± 3.8% vs 58.6 ± 4.3%; 58.3±2.7% vs. 52.4 ± 5.7%, treatment vs. control group). Importantly, however, an additional high dose of atorvastatin (given within 3 to 4 h before the I/R injury) recaptured the protection, reducing infarct size from 58.6 ± 4.3% to 36.2 ± 2.8% after one week and from 51.2 ± 6.7% to 26.8 ± 1.5% after two weeks of oral treatment (Fig. 2).

Western blot for PTEN. After the various treatment regimes with atorvastatin, the level of PTEN in the heart was measured. Gavage with methylcellulose was used as the control. No significant difference was found in PTEN levels (Fig. 3) after acute atorvastatin treatment. After one day and three days of oral administration, the relative densitometry values were 840 ± 230 versus 905 ± 147 and 928 ± 16 versus 837 ± 124 in methylcellulose- and atorvastatin-treated groups, respectively.

Interestingly, when comparing one week of treatment with methylcellulose versus one week of treatment with atorvastatin (Fig. 4), we observed a significant increase in PTEN level (606 ± 149 vs. 1,309 ± 193, respectively, p < 0.05). The same difference could be observed when comparing the two-week methylcellulose treatment versus two-week atorvastatin treatment (3,475 ± 643 vs. 6,383 ± 45, respectively, p < 0.05; Fig. 5).

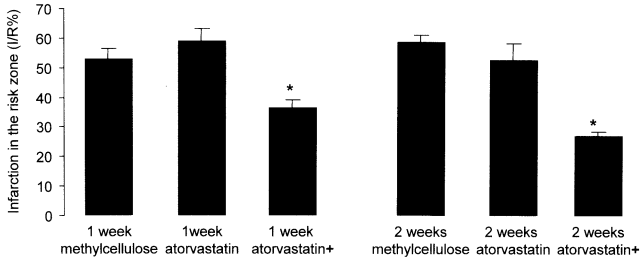

Figure 2. Infarction developed in the risk zone in hearts treated for one or two weeks with atorvastatin or methylcellulose. + = a supplementary dose of atorvastatin given a few hours before ischemia/reperfusion (I/R). *p < 0.05.

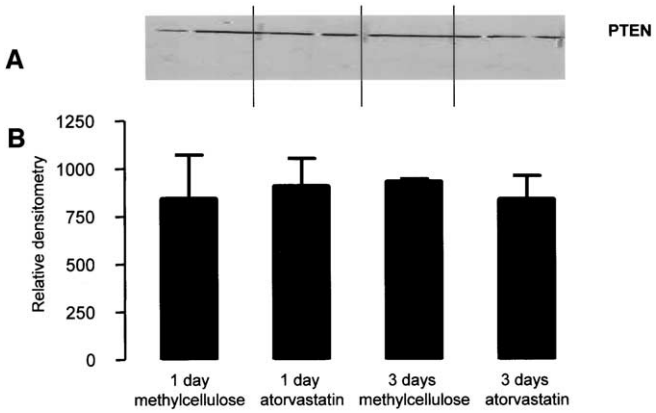

Figure 3. Level of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in hearts treated with methylcellulose or atorvastatin for one or three days. **(A)** Western blots; **(B)** relative densitometry.

DISCUSSION

The results presented in this study show that atorvastatin protects the myocardium after short-term (acute) treatment but that this protection is lost after chronic administration. We have also shown that, using the Western blotting technique, chronic treatment with atorvastatin leads to an increase in the levels of PTEN, the phosphatase that inactivates PI3K. Interestingly, a further dose of atorvastatin, given just before ischemia/reperfusion, restores this protection.

The beneficial effects of statins in the treatment of ischemic heart disease are now well documented, with beneficial effects shown in a number of clinical studies, as previously mentioned (2–5,25). Initially, these effects were thought to be entirely due to cholesterol lowering because there seemed to be a strong correlation between cholesterol levels and adverse effects. However, a number of trials (4,26) have shown beneficial effects in the presence of mildly elevated or even normal cholesterol levels. These results led to the investigation of the other beneficial effects of statins, the so-called, pleiotropic effects. A wide variety of studies have been performed to investigate these effects (27–29),

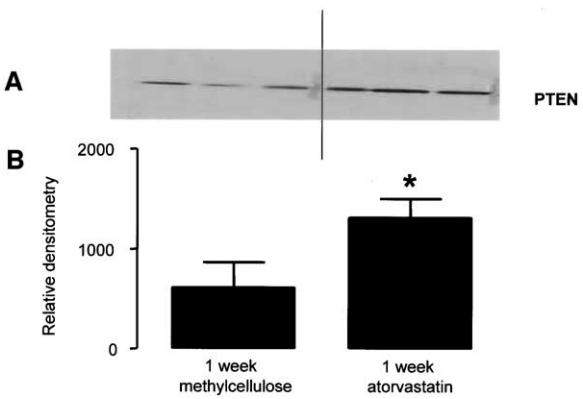

Figure 4. Level of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in hearts treated with methylcellulose or atorvastatin for one week. **(A)** Representative blots; **(B)** relative densitometry. *p < 0.05.

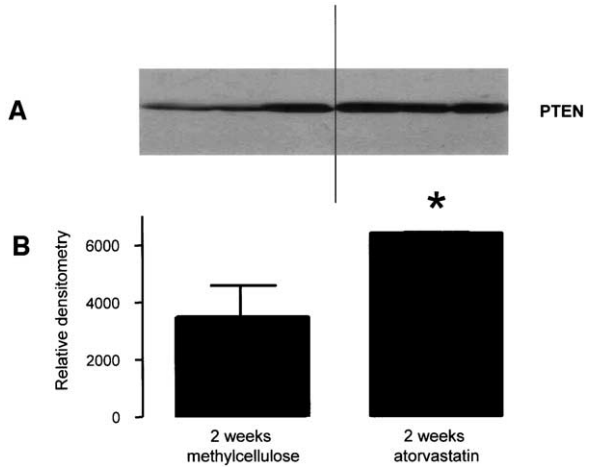

Figure 5. Level of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in hearts treated with methylcellulose or atorvastatin for two weeks. **(A)** Representative blots; **(B)** relative densitometry. *$p < 0.05$.

which include inhibition of smooth muscle proliferation and platelet aggregation, anti-inflammatory effects, and enhancement of endothelial function.

Although most studies have examined endothelial function (for review see, McFarlane et al. [30]), a number of studies have investigated the direct effects of short-term treatment with statins on infarct size and myocardial function in the setting of I/R in the myocardium (9,31). However, in the in vivo techniques the effects upon inflammation, platelet aggregation, and immunomodulation would be expected to play an important part in the protection observed with statins. Surprisingly, statins have been demonstrated to directly activate the PI3K/Akt pro-survival pathway (10,32,33). We have previously demonstrated the importance of PI3kinase and its downstream effector, Akt, as well as endothelial nitric oxide synthase in the protection observed with acute atorvastatin treatment (10). Although we do not observe any protection with chronic atorvastatin treatment, we cannot be sure at this stage whether this is due to a down-regulation of the activity of these kinases, although our studies relating to PTEN may suggest such an effect (as discussed later in this article).

The PI3K/Akt pathway has been shown to be of great importance in the intrinsic protection of the myocardium before ischemia (7,34) and at reperfusion (8), and the activation of this pathway provides an explanation for the protection observed with acute treatment with atorvastatin (35).

From our study, it appears that chronic treatment with atorvastatin does not demonstrate any protection; however, additional acute atorvastatin treatment (on top of chronic) appears to restore this protection. The mechanism underlying this restored protection is at this stage unclear. It is possible that in this setting, other survival pathways that are known to protect the myocardium from I/R injury (35) are also activated. These pathways include the extracellular signal-regulated kinase 1/2, which is also known to exhibit crosstalk with PI3K (36). Alternatively, it could simply be as a consequence of a dose-dependent phenomenon.

Our data relating to the loss of protection, as a result of chronic treatment, are in accordance with other studies (14,37,38), indicating a detrimental effect of long-term statin treatment on myocardial function. Paradoxically, the explanation for this loss of protection with chronic treatment with atorvastatin may also be as a consequence of the ability of statins to activate the PI3K/Akt pathway. As mentioned, the activation of PI3K is beneficial for the cells in short term because, among other effects, it promotes antiapoptotic mechanisms (15–17) through the generation of the second messenger PI-3,4,5-trisphosphate. However, a prolonged activation of PI3K will be associated with cardiomegaly (18–20) or tumor formation (21,22), as has been demonstrated to be the case in many cancers. Therefore, normal cells possess intrinsic mechanisms for limiting chronic activation of PI3K. In this respect, the main suppressor is PTEN, which hydrolyzes PI-3,4,5-trisphosphate in its precursor and PI3K substrate, PI-4,5-diphosphate (39). Interestingly, our Western blot data showed an increase in the levels of PTEN after chronic treatment with atorvastatin in the hearts treated for one and two weeks. Importantly, this increase was associated with a loss of protection after I/R injury. It already has been demonstrated in other cellular systems that PTEN activity is regulated by its protein level (40). Therefore, we would hypothesize that this increase of PTEN may be responsible for the loss of protection observed in the chronic atorvastatin-treated hearts. This important adaptive mechanism, which could account for this loss of protection, may be necessary to regulate growth-inducing kinases.

The pro-survival kinases have been examined in great detail in the setting of preconditioning (7,8,34) and reperfusion-injury salvage (35); however, there is a paucity of literature with regards to the role of phosphatases in this setting. A few studies have investigated the effect of phosphatase inhibitors on myocardial protection (41,42); however, no studies have examined the PI3 kinase-specific phosphatase, PTEN, in the setting of I/R injury. Further detailed studies investigating the importance of PTEN in I/R injury need to be undertaken.

Conclusions. In this work, we present evidence that acute treatment with atorvastatin can protect the myocardium from subsequent I/R injury; however, with chronic or long-term treatment, this protection is lost. Interestingly the protection appears to be recaptured by an increased dose of atorvastatin given 3 to 4 h before the ischemia/reperfusion. We also suggest a potential mechanism that could explain the loss of the protective effect of chronic administration of atorvastatin; this being via an increase in the level of the tumor suppressor, PTEN, which is a ubiquitous regulator of the PI3K activity. Therefore, investigating such a mechanism may provide a new and novel approach in the strategy of statin administration. Moreover, such studies could be

extended to any cardioprotective agent that chronically activates the PI3K/Akt pathway.

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