Genistein and Tyrphostin AG 556 Block the Action Potential Shortening in Septic Shock

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Key Words
action potential; endotoxic shock; nitric oxide; tyrosine kinase

Background. We have previously shown that an increase in NO activity activated ATP-sensitive potassium channel (K_{ATP}) and shortened action potential duration (APD) in an endotoxic shock model. Because the increase in NO production and the decrease of APD appear to be downstream late events in endotoxic shock, we hypothesized that a common signaling pathway might mediate these effects.

Methods. Using a guinea pig model of endotoxic shock, we investigated the effect of genistein and tyrphostin AG 556 on the cardiac action potential. Adult Hartley guinea pigs (300 to 450 gm) were randomized into 2 treatment parts. In the chronic treatment part, guinea pigs were randomized to receive daily subcutaneous injection of one of the five agents: saline, genistein, tyrphostin AG 556, daidzein, and vehicle for 10 days. In the acute treatment part, these agents were administered by intraperitoneal injection 1 hour before endotoxic shock. The animals were then anesthetized and mechanically ventilated, and underwent 6-hour endotoxic shock or sham experiment.

Results. In the chronic treatment part, the plasma nitrate concentration, myocardial guanosine 3',5'-cyclic monophosphate (cGMP) content, and APD at 90% repolarization (APD_{90}) of papillary muscle showed no difference in the five groups before endotoxic shock. After 6-hour endotoxic shock, the elevation of plasma nitrate concentration and myocardial cGMP content was found significant in the control, the daidzein, and the vehicle groups, but was blunted in the genistein and the tyrphostin groups. The shortening of APD_{90} of papillary muscle was also significant in the control, the daidzein, and the vehicle groups, but blunted in the genistein and tyrphostin groups. There were similar findings in the acute treatment part, except the weaker effect of genistein and tyrphostin.

Conclusions. Genistein and tyrphostin AG 556, either administered chronically or acutely, significantly attenuate the cardiac APD shortening in endotoxic shock, presumably through the decrease in the plasma nitrate and the cardiac cGMP production. It is suggested that tyrosine kinase signaling plays an important role in the modulation of APD in endotoxic shock. [Chin Med J (Taipei) 2002;65:570-579]

Septic shock is a major cause of morbidity and mortality in hospitalized patients.¹ It results from a systemic infection by Gram-negative bacteria and presents with hypotension and multiple organ failure. Cardiovascular dysfunction is a grave prognostic sign²,³ that develops in 40% of patients with septic
shock. If it is not treated effectively, ischemic or organ dysfunction and death supervenes.

Nitric oxide (NO) plays important physiological and pathophysiologic roles in cardiovascular systems. During septic shock, myocardial depression and loss of peripheral vascular tone is associated with increased inducible NO synthase (iNOS) activity and NO production. Left ventricular dysfunction and hemodynamic instability could be improved by application of NOS inhibitors both in humans and animals with endotoxemia. These findings suggest the important role of NO in myocardial depression during endotoxic shock, although there are conflicting results.

The contractility of isolated adult rat myocytes was attenuated by exposure to the media from LPS-conditioned macrophages, and the effect could be blocked by pretreatment with NOS inhibitors in humans and animals with endotoxemia. These findings suggest the important role of NO in myocardial depression during endotoxic shock, although there are conflicting results.

Recent studies suggested the involvement of tyrosine kinase signaling in the induction of NOS by endotoxin. However, most of these studies tested acute effects of tyrosine kinase inhibitors on cultured cells. The methodology and findings might not be convincingly applied to electrophysiological study in that the acute direct effects of these agents on the involved ionic channels have to be excluded.

In the present study, using in vivo guinea pig model of endotoxic shock, we demonstrated that both 10-day pre-treatment with acute administration of either genistein or tyrphostin AG 556, but not daidzein (an inactive analogue of genistein), significantly attenuated the APD shortening in the heart with concomitant reduction of NO production and guanosine 3', 5'-cyclic monophosphate (cGMP) production, suggesting the important role of tyrosine kinase signaling in the regulation of cardiac APD, possibly through reduced NO production in endotoxic shock.

### Methods

This study conforms to NIH guidelines for the Care and Use of Laboratory Animals. Throughout the studies, all effects were tested to minimize animal pain and suffering. Adult Hartley guinea pigs (300 to 450 g) of either sex were used in this study. The whole study composed of two parts: a chronic treatment part and an acute treatment part. In the first part (chronic treatment), animals were randomized into 5 groups: control group, genistein group, tyrphostin group, daidzein group, and vehicle (dimethyl sulfoxide, DMSO) group. Animals in each group were further randomized to receive LPS-induced endotoxic shock experiment or sham experiment. Normal saline (0.05 mL/Kg/day), genistein (2.5 mg/Kg/day), tyrphostin AG 556 (2.5 mg/Kg/day), daidzein (2.5 mg/Kg/day), and an equal volume of DMSO (0.05 mL/Kg/day) were injected subcutaneously into anterior abdominal wall of guinea pigs in each group once every day for a total of 10 days. The total cumulative dose of each drug was 25 mg/Kg. The dosage of genistein and tyrphostin have been shown to block tyrosine kinase in previous literatures.

In the second part (acute treatment), animals were also randomized into 5 groups: control group, genistein group, tyrphostin group, daidzein group, and vehicle (dimethyl sulfoxide, DMSO) group. Animals in each group were further randomized to receive LPS-induced endotoxic shock experiment or sham experiment. Normal saline (0.1 mL/Kg), genistein (5 mg/Kg), tyrphostin AG 556 (5 mg/Kg), daidzein (5 mg/Kg), and an equal volume of DMSO (0.1 mL/Kg) were administered intraperitoneally injection in each group 1 hour before LPS injection.
Baseline measurements

For chronic treat ment part, eight guinea pigs were sac ri ficed from each of the 5 groups on the 10th day, 2 hours af ter the in jec tion of the last dose of drugs with out un der going endotoxic shock or sham ex per i ments. Plasma ni trate con cen tra tion, cGMP con tent of ven tri cu lar mus cle, and APD of pap il lary mus cle were mea sur ed or re corded. For the acute treat ment part, seven guinea pigs were sac ri ficed from each group 1 hour be fore drug ad min is tra tion. Plasma ni trate con cen tra tion, cGMP con tent of ven tri cu lar mus cle, and APD of pap il lary mus cle were mea sur ed or re corded. Persons who per formed the baseline measurement were blinded to the ran dom iza tion.

LPS-induced endotoxic shock

Two hours af ter sub cu ta ne ous in jec tion of the last dose of drugs on the 10th day (chronic treat ment part) or af ter the intraperitoneal in jec tion of drugs (acute treat ment part), guinea pigs were an es tived with ure thane (1.2 g/Kg, intraperitoneal in jec tion). Tracheostomy and con trolled ven ti la tion with 90% O₂ were per formed. Ar te rial line was es tab lished in the right ca rotid ar tery for blood sam pling. Fluid was ad min is tered through the right fem o ral vein.

Escherichia coli (E. coli) LPS (serotype 0127:B8, Sigma Chem i cal, St. Louis, MO, USA) was in jected via right fem o ral vein. A dose (10 mg/Kg) enou gh to in duce endotoxic shock within 6 hours was ad min is tered.20,32-37 Sham ex per i ments were per formed in the same way as the LPS-treated ones in terms of an es the sia, tracheostomy, ves sular ac cess, artifi cial ven ti la tion, and du ra tion of study, ex cept LPS ad min is tra tion. Per sons who per formed the LPS-induced endotoxic shock ex per i ments or sham ex per i ments and who mea sured the plasma ni trate con cen tra tion, the cGMP con tent and APD of the pap il lary mus cles were blind to the ran dom iza tion.

Determination of plasma nitrate concentration

Immediately before LPS injection and after 6 hours’ endotoxic shock, plasma ni trate con cen tra tion was mea sured in all 5 groups in both the chronic treat ment part and the acute treat ment part. The de tailed method for an a lyzing plasma ni trate con cen tra tion has been re ported pre vi ously.20

Determination of cGMP content of ventricular tissue

In both the chronic treat ment part and the acute treat ment part, cy clic GMP con tent of ven tri cu lar tis sue was mea sured in the base line con di tion as men tioned above. It was also measured after 6-hour endotoxic shock or 6-hour sham ex per i ment. Thus, they were mea sured from differ ent an i mals. Guinea pigs were sac ri ficed by cer vi cal dis lo ca tion and the heart was ex cised im me di ate ly. All the ven tri cu lar mus cles ex cept the pap il lary mus cles were dis sec ted and col lected, and im me di ate ly frozen in a con tainer in liq uid ni tro gen (-80 ºC). The method for mea sur ing the cGMP con tent has been re ported.20

Recording of cardiac action potentials

In both the chronic treat ment part and the acute treat ment part, cardiac action potentials were re corded with con ven tional re cord ing tech nique20 from the pap il lary mus cles in the base line con di tion as men tioned above and also after 6-hour endotoxic shock or 6-hour sham ex per i ment. Thus, they were mea sured from differ ent an i mals, be cause pap il lary mus cles could only be ob tained af ter an im al sac ri fic e. Only one pap il lary mus cle was har vested from each guinea pig, i.e., the “n” is the num ber of pap il lary mus cles and there fore the num ber of guinea pigs. In brief, a strand of free-run ning pap il lary mus cle from right ven tri cle was care fully dis sec ted and im me di ate ly mounted into a 2-mL per fu sion cham ber. The per fu sion speed was set at 5 ml/min. The com po si tion of nor mal Tyrode so lu tion and the method for the re cord ing of APD have been re ported pre vi ously.20
Drugs and data analysis

*E. coli* LPS (serotype 0127:B8), genistein, and daidzein were purchased from Sigma (St. Louis, MO, USA). Tyrphostin AG 556 was purchased from Calbiochem (La Jolla, CA, USA). Lipopolysaccharide was dissolved in normal saline, while other agents were dissolved in DMSO. The final concentration of DMSO in the perfusate was < 0.1%.

All values are expressed as mean ± S.D. We used a computer-based statistical package (SPSS version 9.0, SPSS Inc., Chicago, IL, USA) to perform two-way ANOVA to analyze the experimental data, with one way being between groups and the other way being within group. If significance was found, a post hoc analysis (Newman-Keuls) was used. The difference was considered statistically significant when p value was less than 0.05.

Results

Baseline data after 10-day drug treatment

In the chronic treatment part, no an i mals died during the 10-day drug treatment. The base line data after 10-day drug treatment are summarized in Table 1. Plasma nitrate concentration and myocardial cGMP content did not differ significantly among the 5 groups. Neither did the APD at 90% repolarization (APD$_{90}$) show any significant difference.

Effects on plasma nitrate concentration

Figure 1A shows the plasma nitrate concentration after 6-hour endotoxic shock in the chronic treatment part of the study. Among all the LPS-treated an i mals, the plasma nitrate concentration was significantly lower in the genistein group ($p < 0.01$ versus the control group, $p < 0.01$ versus the daidzein group, $p < 0.01$ versus the DMSO group) and the tyrphostin group ($p < 0.01$ versus the control group, $p < 0.01$ versus the daidzein group, $p < 0.01$ versus the DMSO group). There was no significant difference between the genistein and the tyrphostin groups ($p > 0.05$). In addition, there was no significant difference among the control group, the daidzein group and the DMSO group ($p > 0.05$).

Among the genistein group, the plasma nitrate concentration of the LPS-treated an i mals was higher than the base line value ($p < 0.05$) and that of the an i mals receiving the sham experiment ($p < 0.05$). There were similar findings in the tyrphostin group.

In the control group, the plasma nitrate concentration of the LPS-treated an i mals was markedly higher than the base line value ($p < 0.01$) and that of the an i mals receiving the sham experiment ($p < 0.01$). Similar findings were observed in the daidzein group and the DMSO group. In all the 5 groups, the sham experiment did not significantly affect the plasma nitrate concentration as compared with the base line value (all $p > 0.05$ versus base line value).

Figure 1B shows the plasma nitrate concentration after 6-hour endotoxic shock in the acute treatment part of the study. The findings were similar to those in the chronic treatment part. The difference was in the genistein group and the tyrphostin group. The acute treatment part showed less degree of suppression of the plasma nitrate concentration compared with the chronic treatment group for both genistein and tyrphostin (24.6 ± 4.8% vs. 39.1 ± 6.2% for genistein, $p < 0.05$; 25.1 ± 2.9% vs. 40.9 ± 4.2% for tyrphostin, $p < 0.05$).

Table 1. Baseline data after 10-day drug treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Genistein</th>
<th>Tyrphostin</th>
<th>Daidzein</th>
<th>DMSO</th>
<th>p value</th>
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<tr>
<td>Nitrate (µM)</td>
<td>28.8 ± 6.3</td>
<td>28.5 ± 7.1</td>
<td>28.4 ± 4.9</td>
<td>29.0 ± 6.8</td>
<td>28.4 ± 5.8</td>
<td>NS</td>
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<tr>
<td>cGMP (pmol/mg protein)</td>
<td>0.72 ± 0.25</td>
<td>0.74 ± 0.22</td>
<td>0.76 ± 0.31</td>
<td>0.73 ± 0.35</td>
<td>0.78 ± 0.35</td>
<td>NS</td>
</tr>
<tr>
<td>APD$_{90}$ (msec)</td>
<td>186.5 ± 6.7</td>
<td>184.2 ± 6.2</td>
<td>184.9 ± 5.1</td>
<td>183.1 ± 4.8</td>
<td>183.4 ± 3.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = non-significant; DMSO = dimethyl sulfoxide; cGMP = 3',5'-cyclic monophosphate; APD$_{90}$ = action potential duration at 90% repolarization.
Effects on myocardial cGMP content

Figure 2A shows the changes in the myocardial cGMP content in the chronic treatment part of the study. Among all the LPS-treated animals, the myocardial cGMP content was significantly lower in the genistein group (p < 0.05 versus the control group, p < 0.05 versus the daidzein group, p < 0.05 versus the DMSO group) and the tyrphostin group (p < 0.05 versus the control group, p < 0.05 versus the daidzein group). There was no significant difference between the genistein and the tyrphostin groups (p > 0.05), neither among the control group, the daidzein group, and the DMSO group (p > 0.05).

In the genistein group, the myocardial cGMP content of the LPS-treated animals was higher than the base line value (p < 0.05) and that of the animals receiving the sham experiment (p < 0.05). There were similar findings in the tyrphostin group.

In the control group, the myocardial cGMP content of the LPS-treated animals was markedly higher...
than the base line value ($p < 0.01$) and that of the animals receiving the sham experiment ($p < 0.01$). Similar findings were observed in the daidzein group and the DMSO group. In all the 5 groups, the sham experiments did not significantly affect the myocardial cGMP content as compared with the base line value (all $p > 0.05$ versus base line value).

Figure 2B shows the changes of cGMP content in the acute treatment part of the study. The findings were similar to those in the chronic treatment part. The difference was in the genistein group and the tyrphostin group. The acute treatment part showed less degree of suppression of the increase in the myocardial cGMP content than the chronic treatment part for both genistein and tyrphostin (38.9 ± 5.2% vs. 64.1 ± 7.2% for genistein, $p < 0.05$; 37.6 ± 3.5% vs. 62.6 ± 5.4% for tyrphostin, $p < 0.05$).

**Effects on the APD of papillary muscle**

The changes in the APD$_{90}$ are shown in Figure 3. In the chronic treatment part (Fig. 3A), APD$_{90}$ shortened significantly after 6-hour endotoxemia in the control group ($p < 0.01$ versus the base line value; $p < 0.01$ versus the sham experiment). Ten-day treatment with genistein or tyrphostin significantly abolished the APD-shortening effect of endotoxic shock (both $p < 0.01$ versus control group; $p > 0.05$ between each), but the APD was still shorter than the base line value and that of the sham experiment in the same group (both $p < 0.05$). Daidzein and DMSO had no effect on the APD-shortening phenomenon (both $p > 0.05$ versus control group). The sham experiment had no significant effect on the APD$_{90}$ in all the 5 groups (all $p > 0.05$ versus the base line values). Figure 3B shows the changes in APD$_{90}$ in the acute treatment part of the study. The only difference was in the genistein and the tyrphostin groups. The APD$_{90}$ was shorter in the acute treatment part than that in the chronic treatment part (154.2 ± 10.4 msec vs. 168.0 ± 5.7 msec for genistein, $p < 0.05$; 152.8 ± 12.0 msec vs. 164.0 ± 6.9 msec for tyrphostin, $p < 0.05$), suggesting a stronger effect in the chronic treatment part than in the acute treatment part. Figure 4 shows representative illustrations of the action potentials recorded in the base line condition and after 6-hour endotoxemia in the chronic treatment part.

**Discussion**

The main findings in this *in vivo* model of endotoxemia are that genistein and tyrphostin AG 556 significantly attenuate the cardiac APD shortening...
with concomitant decrease in the plasma nitrater concentration and the cardiac cGMP content. The inactive analogue of genistein and the vehicle have no effects. These findings suggest an important role of tyrosine kinase signaling in the modulation of APD in endotoxic shock.

Tyrosine kinase inhibitors prevented the LPS-induced lethal toxicity in mice.\(^\text{38}\) In canine \textit{E. coli} peritonitis, it improved survival and reduced multiorgan failure.\(^\text{31}\) Inhibition of tyrosine kinase signaling has also been reported to attenuate the vascular hyporesponsiveness in endotoxic shock.\(^\text{39}\) However, this is the first study to demonstrate that tyrosine kinase in hi bition has re ver sal effect in the APD shortening in endotoxic shock.

A recent study showed that cardiac contractility was not depressed in early (\(\leq 4\) hours) canine endotoxic shock.\(^\text{40}\) However, other studies demonstrated that endotoxic shock in deed impaired LV function\(^\text{41}\) and shortened cardiac APD.\(^\text{19,20}\) The degree of the shortening of APD positively correlated with the amount of NO produced.\(^\text{20}\) Inducible NOS in duction is regulated by multiple signaling pathways in cultured cardiac myocytes,\(^\text{42}\) and ty ro sine kinase pathway has been shown to be involved in the early signaling cascade of LPS in a number of cellular studies.\(^\text{43}\) It seems to be true in the whole animal study as well, as shown in the present study.

Mechanisms responsible for the shortening of APD in endotoxic shock remain debate able. In rabbits, the APD shortening in endotoxic shock is mainly related to endotoxic-mediated sarcoplasmic reticulum alterations, in stead of alterations in sarcoplasmic reticulum function.\(^\text{19}\) Other investigators demonstrated that after 4-hour endotoxemia in guinea pigs the isolated ventricular myocytes had diminished sarcoplasmal L-type Ca\(^{2+}\) current.\(^\text{44}\) We found that glibenclamide could re verse the APD shortening after 6-hour endotoxemia in guinea pigs and proposed that activation of sarcoplasmal K\(_{\text{ATP}}\) channels plays a major role in the shortening of APD.\(^\text{20}\) Protein phosphorylation at the tyrosine residues has been shown to be involved in the modulation of different kinds of voltage-gated ion channels in the myocytes,\(^\text{45}\) in cluding Na\(^{+}\) channels,\(^\text{26}\) Ca\(^{2+}\) channels,\(^\text{46}\) K\(^{+}\) channels\(^\text{47,48}\) and other ligand-gated ion channels such as cystic fibrosis transmembrane regulator Cl channels.\(^\text{49}\) Tyrosine kinase signaling also plays an important role in the modulation of mi tochondrial K\(_{\text{ATP}}\) channels in the ischemic preconditioning,\(^\text{50}\) and pretreatment with genistein suppressed the APD shortening during myocardial ischemia and preconditioning.\(^\text{51}\) We proposed that during endotoxic shock tyrosine kinase signaling is involved in the modulation of sarcoplasmal K\(_{\text{ATP}}\) channel. Since the shortening of APD was not completely prevented by genistein and tyrphostin, it is possible that other unknown mechanisms are involved or that the inhibition of tyrosine kinase is incomplete. It remains to be determined whether the activation of tyrosine kinase signaling increases the NO production to further enhance the K\(_{\text{ATP}}\) channel activity or directly phosphorylates tyrosine residues of the K\(_{\text{ATP}}\) channels.

In the study of tyrosine kinase signaling path way by using an antagonist, it is important to exclude the acute direct effects of these agents.\(^\text{52,53}\) In our chronic treatment part, we administered these agents for 10 days. Thereafter, we examined the parameters before the induction of endotoxic shock. We found that the NO production and the APD of the heart were simi lar to control.
among all groups. Thus, the acute direct effects of these agents could be excluded. Furthermore, it is possible that tyrosine kinase signaling pathway is not involved in the basal NO production and the regulation of APD in the resting state. On the other hand, the effects of genistein and tyrphostin were greater in the chronic treatment part than in the acute treatment part, suggesting the cumulative effect of these agents. In the present study, we measured the APD by conventional microelectrode recording technique in the right ventricular papillary muscle of guinea pig instead of measuring the monophasic action potential in vivo or the APD in single isolated myocytes. Others and we have demonstrated that the changes in APD of endotoxic shock could persist in the papillary muscle after its dissection from the animals. 

Nitric oxide is very unstable and rapidly oxidized in aqueous solution. It may release followed by formation of the stable oxidative metabolites nitrite, which is itself oxidized in vivo to nitrate, an even more stable end product of NO. The plasma nitrate concentration measured in this study is the total nitrite and nitrate concentration. Although it is a balance between its production and excretion, increased production of NO is a more favored mechanism during endotoxic shock. 

The methodology used in this study does not allow for determination of NOS enzyme activities, nor for identification of cells responsible for NO production. However, these were not the purpose of this study. Besides, their enhancement may represent an increase in the capacity of NO production, not necessarily an actual increase in NO production. We in deed observed an increase in the plasma nitrate concentration and an increase in car diaic cGMP content in the control group, and the increase could be blunted by genistein and tyrphostin.

The base line data of myocardial cGMP content and the APD of the papillary muscles, and those after 6-hour experiment were measured from different animals because they could only be measured after animal sacrifice. 

We did not measure the phosphotyrosine content in the heart or in other tissues. There are multiple possible targets for the tyrosine phosphorylation, and it remains to be determined which one plays a major role in the regulation of APD in the heart. One might also argue that these tyrosine kinase inhibitors did not exert effects. However, the inactive analogue, daidzein, had no protective effect at all, suggesting the involvement of tyrosine kinase.

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