Influence of a Pyrimidopyrimidine Derivative on Deamination of Adenosine by Blood

By Ruben D. Bunag, M.D., C. Roberto Douglas, M.D., Shoichi Imai, M.D., Ph.D., and Robert M. Berne, M.D.

A new synthetic compound, Persantin (RA-8 or 2,6-Bis(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidine), has recently been reported to produce coronary vasodilation without an associated increase in cardiac work. Although the mechanism responsible for the coronary vasodilation produced by Persantin has not been elucidated, there are some indications that an indirect action on myocardial nucleoside metabolism may be involved. In anesthetized dogs, it has been shown that the administration of Persantin results in an increase in ATP content of the myocardium and potentiation of adenosine-induced coronary vasodilation. More recently, it was observed that the disappearance of adenosine in shed blood is prevented by the addition of Persantin.

A theory involving adenosine mediation has previously been proposed to explain the metabolic regulation of coronary blood flow. According to this hypothesis, conditions such as hypoxemia, diminished coronary blood flow or increased oxygen utilization by the myocardium result in a reduction of myocardial oxygen tension which in turn leads to a breakdown of adenine nucleotides to adenosine. The adenosine that is formed then diffuses out of the cell and, via the interstitial fluid, reaches the coronary arterioles to produce dilation. If the action of Persantin also involves chemical mediation by adenosine, then this drug may prove to be a useful tool with which the more complex nature of coronary autoregulation can be explored. With this relationship in mind, the present studies were carried out to determine the mechanism by which Persantin affects nucleoside metabolism.

Methods

Two procedures were employed to assess the effect of Persantin on adenosine deaminase activity. In four experiments, the time course of the decrease in absorption at 265 nm, resulting from the addition of adenosine deaminase to a solution of the substrate, was determined. The change in optical density with time was continuously recorded in the absence and presence of Persantin and 8-azaguanine, a known inhibitor of adenosine deaminase. In a second series of experiments, the effect of Persantin on the recovery of adenosine from buffered, aqueous solutions was studied. Adenosine, 0.1 to 0.2 μmole, was added to test tubes containing 2 ml of 0.05 M potassium phosphate buffer, pH 7.4. Duplicate samples contained various concentrations of Persantin or 8-azaguanine. Adenosine deaminase (750 units) was added to each tube and all samples were allowed to stand at room temperature for five minutes. Enzymatic activity was terminated by immersing the tubes in a boiling water bath and aliquots were then taken from each tube for adenosine analysis.

Experiments were also performed on samples of fresh, heparinized blood obtained from dogs or human subjects. Prior to use, the blood was diluted with isotonic saline for the experiments on whole blood or with water for the studies on hemolyzed blood. In the experiments on dog blood, suspensions of washed red cells were used.

* Persantin was generously supplied by Geigy Pharmaceuticals, Ardsley, New York.

† Alkaline phosphatase (intestine) which contains adenosine deaminase was purchased from Nutritional Biochemicals Corporation and was treated and used for assay of adenosine in the manner described by Kornberg and Fricer.
The samples were incubated at 30°C and adenosine, 0.03 to 0.3 μmole/ml, was added to zero time as described by Koss et al. One ml aliquots were taken at periodic intervals and deproteinized with equal volumes of 0.6 N perchloric acid. The resulting precipitates were washed with 0.5 ml of 0.2 N perchloric acid and the washings added to the initial supernatants. The pH of the samples was adjusted to seven with potassium hydroxide and the potassium perchlorate precipitate was removed by centrifugation. Sample volumes were adjusted to 3 ml by the addition of water and aliquots were then analyzed for nucleoside content. Simultaneous determinations were made on duplicate samples containing Persantin or 8-azaguanine in various concentrations. Similar experiments were conducted on blood samples without the addition of adenosine and on samples to which equimolar amounts (0.3 μmole/ml) of inosine and hypoxanthine were added instead of adenosine. Assays for adenosine, inosine, and hypoxanthine were performed by the enzymatic methods of Kalckar.7,8

Changes in optical density were measured with a Gilford model 220 absorbance indicator attached to a Beckman model DU spectrophotometer and recorded with a modified Minneapolis-Honeywell recorder. Because of the impurity of the nucleoside phosphorylase (prepared from rat liver), assays for inosine were conducted by initially analyzing the samples for adenosine content and adding adenosine deaminase (3,000 units) to each sample in order to deaminate all the adenosine present. The total amount of inosine was determined and the original inosine content of the sample calculated by difference.

**Results**

**ADENOSINE DEAMINASE INHIBITION**

The time course of the decrease in absorption at 265 μm resulting from the deamination of adenosine in buffered, aqueous solutions is graphically presented in figure 1. In the presence of Persantin or 8-azaguanine, there was a diminution in both the slope of the curve and the maxima attained, indicating deceleration of the rate of deamination.

In the experiments in which the amount of adenosine was measured after incubation of the enzyme and substrate at room temperature an average of 14 ± 10% of the 0.1 to 0.2 μmole of added adenosine remained. Upon addition of Persantin or 8-azaguanine in concentrations of 10⁻⁴ M or more, the amount of residual adenosine recovered was increased. The data given in table 1 are expressed in terms of per cent inhibition of adenosine deaminase activity as computed from controls containing only the substrate-enzyme system. It is evident that when Persantin and 8-azaguanine were used in the indicated concentrations in a buffered, aqueous medium, adenosine deaminase activity was significantly inhibited.

**RECOVERY OF ADENOSINE FROM BLOOD**

The rate at which added adenosine disappeared from a suspension of washed canine erythrocytes in the presence and absence of Persantin is shown in figure 2. In the absence of Persantin a progressive disappearance of adenosine occurred with time, whereas in the presence of Persantin the loss of adenosine was greatly retarded. No significant effect on the loss of exogenous adenosine from suspensions of dog red blood cells was observed with
PYRIMIDOPYRIMIDINE AND ADENOSINE DEAMINATION

Effects of Persantin on recovery of adenosine from a 10% suspension of washed dog red blood cells in isotonic sodium chloride solution. Solid line = control; broken line = Persantin in concentration of $1 \times 10^{-3}$ M. Adenosine concentration 0.03 $\mu$ mole/ml.

Figure 3 represents the results obtained from similar experiments carried out with human blood. In contrast to what was observed with dog blood, exogenous adenosine disappeared with equal rapidity from hemolyzed and whole human blood. In concentrations up to $10^{-4}$ M, 8-azaguanine did not affect the rate of adenosine disappearance from whole or hemolyzed human blood and equimolar concentrations of Persantin were ineffective in hemolyzed blood. However, in whole human blood, the loss of added adenosine was effectively prevented by Persantin in concentrations of $10^{-6}$ to $10^{-4}$ M.

Most of the exogenous adenosine (67 to 100%) which gradually disappeared in whole blood could be accounted for by the appearance of inosine and hypoxanthine in the sample. The data presented in table 2 were obtained from an experiment in which samples were assayed for adenosine, inosine, and hypoxanthine following the addition of adenosine (0.3 $\mu$ mole/ml) to whole blood. In the absence of any drugs, the disappearance of adenosine with time was associated with a corresponding increase in the amounts of inosine and hypoxanthine recovered. In the presence of Persantin, adenosine values remained elevated and the recoveries of inosine and hypoxanthine were correspondingly diminished. Within the dose range employed, there is a direct relationship between the dose of Persantin used and the adenosine-sparing effect produced.

When assays were made on whole human blood without previous addition of exogenous nucleosides, small amounts of endogenous adenosine (0 to 0.034 $\mu$ mole/ml), but not of inosine or hypoxanthine, were detected. These findings were not affected by Persantin in concentrations up to $10^{-4}$ M. When inosine and hypoxanthine were added to the blood in place of adenosine, the levels of the added substances were maintained unchanged throughout the 30-minute period of observation.

CHARACTERIZATION OF THE ADENOSINE-SPARING EFFECT

To study the characteristics of the adenosine-sparing effect of Persantin in whole human blood, experiments were done in which various concentrations of the drug were preincubated
TABLE 2

Effects of Persantin * on Recovery of Adenosine, Inosine, and Hypoxanthine from Whole Human Blood

<table>
<thead>
<tr>
<th>Additions</th>
<th>Time in minutes</th>
<th>Adenosine</th>
<th>Inosine</th>
<th>Hypoxanthine</th>
<th>Total</th>
</tr>
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<tr>
<td>None</td>
<td>0.5</td>
<td>0.263</td>
<td>0.024</td>
<td>0.020</td>
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<td>0.046</td>
<td>0.241</td>
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<td>0.013</td>
<td>0.219</td>
<td>0.051</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.005</td>
<td>0.234</td>
<td>0.089</td>
<td>0.298</td>
</tr>
<tr>
<td>Persantin</td>
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<td>0.246</td>
<td>0</td>
<td>0.031</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
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<td>0.158</td>
<td>0.025</td>
<td>0.018</td>
<td>0.201</td>
</tr>
<tr>
<td>1 x 10^{-6} M</td>
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<td>0.112</td>
<td>0.004</td>
<td>0.039</td>
<td>0.215</td>
</tr>
<tr>
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<td>0.086</td>
<td>0.084</td>
<td>0.055</td>
<td>0.225</td>
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<tr>
<td></td>
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<td>0.086</td>
<td>0.123</td>
<td>0.050</td>
<td>0.239</td>
</tr>
<tr>
<td>Persantin</td>
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<td>0.249</td>
<td>0</td>
<td>0.010</td>
<td>0.259</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.226</td>
<td>0.018</td>
<td>0.002</td>
<td>0.246</td>
</tr>
<tr>
<td>1 x 10^{-6} M</td>
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<td>0.231</td>
<td>0.016</td>
<td>0</td>
<td>0.247</td>
</tr>
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<td></td>
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<td>0.057</td>
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</tr>
<tr>
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<td>0.061</td>
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<td>Persantin</td>
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</table>

* RA-8 or 2,6-Bis(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidine.
† Blood was diluted 1:4 in isotonic saline solution and 0.3 μmole/ml of adenosine was added at zero time.

Discussion

The brevity of the cardiovascular effects of adenosine has been ascribed to its rapid deamination.10 Therefore the potentiation of adenosine-induced coronary vasodilation3 by Persantin can conceivably be explained on the basis of adenosine deaminase inhibition by the drug. It has been shown that 8-azaguanine

Circulation Research, Vol. XV, July 1964

at 30°C for 30 to 60 minutes with either aqueous solutions of adenosine or suspensions of red blood cells. Preincubation of the drug with adenosine prior to addition to blood did not enhance the adenosine-sparing effect. In two experiments, mixtures containing Persantin and adenosine were preincubated for one hour and then added to samples of whole blood. The protective effect observed with these preincubated mixtures was the same as that obtained when Persantin and adenosine were added to the blood separately. In solutions containing mixtures of Persantin and adenosine, Persantin could be extracted with chloroform without affecting the amount of adenosine present. When preincubated mixtures of Persantin and adenosine were extracted with chloroform and then added to whole blood, adenosine disappeared just as rapidly as in control experiments in which Persantin had not been added.

When Persantin, in a concentration of 10^{-6} M, was preincubated with a red blood cell suspension for 30 minutes and the cells subsequently washed with saline until the washings were free of any aqueous soluble Persantin, as evidenced by the absence of a yellow color, a sufficient amount of Persantin still remained with the red cells to produce an adenosine-sparing effect. The residual Persantin present under these experimental conditions (20% of the added Persantin) could not be extracted with chloroform.
inhibits adenosine deaminase activity in vitro and the present data indicate that a similar enzymatic inhibition can be produced with equal concentrations of Persantin. The failure of Persantin or 8-azaguanine to inhibit adenosine deaminase as effectively in hemolyzed blood as in an aqueous solution may have been due to interference of proteins or other substances present in the plasma or lysed cells. Concentrations of Persantin greater than $4 \times 10^{-4}$ M could not be tried because of precipitation of the drug at or near neutral pH. However, adenosine deaminase inhibition does not appear to be involved in the adenosine-protection produced by Persantin in whole blood. The adenosine-sparing effect of Persantin in whole blood was evident even with drug concentrations as low as $10^{-4}$ M confirming the observation of Koss et al. The fact that 8-azaguanine, used in concentrations as high as $10^{-4}$ M, did not affect the disappearance of exogenous adenosine from either hemolyzed or whole blood argues against the possibility of a mechanism based on adenosine deaminase inhibition.

When exogenous adenosine gradually disappeared from whole blood, there was a corresponding increase in the amounts of inosine and hypoxanthine recovered. This finding indicates that the disappearance of exogenous adenosine from blood is due to its deamination within erythrocytes to form inosine followed by subsequent cleavage of the ribose moiety to produce hypoxanthine. In the presence of Persantin the process of deamination is evidently prevented, as shown by the fact that the sustained high levels of adenosine are accompanied by low and relatively stationary levels of inosine and hypoxanthine. The possibility that the high blood adenosine level maintained in the presence of Persantin is due to degradation of endogenous adenine nucleotide is ruled out by the observation that adenosine is virtually absent in normal dog and human blood in the absence or presence of Persantin.

The disappearance rate of adenosine added to whole blood depends on the amount of adenosine deaminase present in the cells and on the permeability of the erythrocyte membrane to adenosine. It has been postulated that Persantin acts by reducing the permeability of the red cell membrane to adenosine. An alternative explanation would be that Persantin combines with adenosine to form a loose complex which cannot be transported across the red cell membrane. The absence of an effect of preincubation on the action of Persantin and the fact that the effective form of the drug can still be extracted with chloroform even after its preincubation with adenosine, does not support the concept of a Persantin-adenosine complex. The finding that Persantin cannot be extracted with chloroform following preincubation of the drug with a red cell suspension indicates that the site of action involved is not the substrate but the erythrocyte. Regardless of the basic mechanism involved, it is evident that in the presence of intact red blood cells, Persantin prevents the deamination of exogenous adenosine in blood.

Exogenous adenosine added to hemolyzed dog blood disappeared at a rapid rate, indicating the existence of a high degree of adenosine deaminase activity. Since the rate of disappearance became markedly retarded in whole dog blood even without the addition of any inhibitory drugs, it would appear that the red cell membrane in this species is less permeable to adenosine than that of human red cells. It is therefore more difficult to demonstrate the adenosine-sparing effect of Persantin in whole dog blood. However, with low concentrations of adenosine, as may exist in cardiac venous blood during myocardial hypoxia, even this reduced degree of permeability is probably sufficiently great to prevent detection of adenosine in coronary sinus blood. In human red blood cells, the membrane is highly permeable to adenosine and the protective action of Persantin is correspondingly easy to demonstrate.

Although adenosine has been suggested as the chemical mediator involved in the regulation of coronary blood flow, its presence in coronary effluents has not been proven. When isolated hearts were perfused with adenosine, the nucleoside produced an increase in coronary blood flow with a concomitant increment in the amounts of inosine and hypoxanthine.
in the perfusate. Similarly in hearts subjected to severe hypoxia in vivo, the coronary vasodilation produced was accompanied by an increase in the inosine and hypoxanthine content of coronary sinus blood. These findings suggest that adenine nucleotide degradation may be causally related to the vasodilation observed with hypoxia and that under normal conditions coronary blood flow is controlled by a fine balance between the production and the destruction and/or washout of a vasodilator substance like adenosine. The failure to demonstrate the presence of adenosine under these various experimental situations can be attributed to its rapid deamination in blood and myocardial tissue. Under more physiological conditions the amounts of adenosine released, though still sufficient to produce vasodilation, are possibly even smaller and the technical difficulties involved in separation and enzymatic identification and quantitation are further magnified. Since the present data show that Persantin effectively prevents the deamination of adenosine in blood, this drug promises to be a valuable tool in studying the role of adenine nucleotide derivatives in the regulation of coronary blood flow.

Summary

The disappearance of exogenous adenosine from blood is principally due to its degradation into inosine and hypoxanthine by the enzymes present in the erythrocytes. In the presence of intact red cells, Persantin (RA-8 or 2,6-Bis(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidine) effectively prevents the disappearance of adenosine. Although Persantin inhibits adenosine deaminase its adenosine-sparing action in whole blood is more likely due to a reduction in permeability of the red cell membrane to adenosine. This property of Persantin makes possible its use as a tool in investigation of the role of adenine nucleotide derivatives in the regulation of the coronary circulation.

Acknowledgment

The authors gratefully acknowledge the excellent technical assistance of Miss M. Hadady.
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Circ Res. 1964;15:83-88
doi: 10.1161/01.RES.15.1.83

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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