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Protective Effects of Clopidogrel on Oxidant Damage in A Rat Model of Acute Ischemia

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Kanko, M., Maral, H., Akbas, M.H., Ozden, M., Bulbul, S., Omay, O., Yavuz, S. and Berki, K.T. *Protective Effects of Clopidogrel on Oxidant Damage in A Rat Model of Acute Ischemia*. Tohoku J. Exp. Med., 2005, **205** (2), 133-139 —— Reperfusion injury is a consequence of inadequate energy supply and acidosis in ischemic tissues and a chain of events triggered by oxygen-derived free radicals released in response to exposure of oxygen. In this study, we aimed to assess the effects of clopidogrel, an antithrombotic agent, on experimental ischemia-reperfusion model in rats. The ischemia was performed by blockade of the circulation of right lower extremity at trochanter major level for 6 hours. Then, the extremity was reperfused for 4 hours. Another group of rats pretreated with clopidogrel (0.2 mg/kg/day) for 10 days prior to ischemia-reperfusion. After the reperfusion period, all rats were anesthetized with ketamine. Blood and tissue samples from the gastrocnemius muscle, liver and lungs were taken for the measurement of malondialdehyde (MDA), glutathione (GSH) levels and superoxide dismutase (SOD) activity. The results revealed that clopidogrel prevented the increase in MDA level and the decrease in GSH level and SOD activity caused by ischemia-reperfusion both in tissue samples and plasma. These findings suggest that clopidogrel is beneficial in prevention of ischemia-reperfusion injury probably via its effects on inflammatory cells, platelets, and endothelial cells. ———— oxidant; lipid peroxidation; ischemia-reperfusion; clopidogrel

Acute ischemia of the extremities is a major clinical problem which may lead to serious consequences. The risk of mortality and morbidity remains to be of clinical importance following diagnosis and treatment of the acute phase (Becquemin and Ernenwein 1990). Even if complete reperfusion can be provided, loss of extremity, acute renal failure, impairment of respiratory functions and cardiac and neurological disorders may still develop. This clinical state is known as "myo-nephropathic metabolic syndrome" (Haimovici 1996) and is the main cause of mortality and morbidity especially when reperfusion is delayed. Despite advances in medical and surgical approaches, this clinical syndrome remains to be a serious problem.

Ischemia-reperfusion injury is a consequence of inadequate energy supply, acidosis in ischemic tissues, and a chain of events, which may result in cellular death caused by free oxygen radicals released in response to exposure of oxygen (Hearse 1990). Several studies have shown that reactive

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oxygen species (ROS) play a crucial role in the initiation and progression of a wide range of diseases including ischemia-reperfusion injury (Schraufstatter and Cochrane 1997; Mc Cord 2000). ROS are believed to cause lipid peroxidation resulting in damage to biological membranes. Cells have a complete set of antioxidant mechanisms that defense the tissues against the damaging effects of free radicals. Endogenous antioxidants, glutathione (GSH) and superoxide dismutase (SOD) are among the first line of defense mechanisms. They act by scavenging potentially damaging free radical moieties (Freeman and Crapo 1982; Cheeseman and Slater 1993). Previous studies have shown that various pharmacological agents such as mannitol, ascorbic acid, vitamin E, flavanoids and allopurinol suppress the effects of ROS and improve metabolic imbalances (Smeets et al. 1995; Shah et al. 1996). End products of lipid peroxidation in reperfusion period are mainly aldehydes, hydrocarbon gases, and malondialdehyde. It is difficult to quantitate the ROS because of their reactive nature and short lives; thus, the measurement of malondialdehyde level -end product of lipid peroxidation- can be used to estimate the extent of oxidant damage in tissues (Draper and Hadley 1990).

Clopidogrel is a thioenopyridine-derived antiplatelet drug which has been used for the management of chronic ischemia. The antiplatelet effect of the drug results from antagonism of a platelet ADP receptor, P2T, and results in inhibition of platelet activation and aggregation. This antagonism is non-competitive, irreversible, and results in 50-70% inhibition of platelet fibrinogen binding. Previous studies have demonstrated that clopidogrel reduces formation of both arterial (Hechler et al. 1998) and venous (Herbert et al. 1993) thrombi. Therefore, in the present study, we aimed to investigate whether clopidogrel would restore or at least decrease the harmful effects of ischemia-reperfusion via its antiaggregant effects.

Materials and Methods

Animals

This study was conducted in compliance with the guidelines of the National Institutes of Health Guidelines of Care and Use of Laboratory Animals, and approved by the Ethic Committee of Kocaeli University School of Medicine. Male adult Sprague-Dawley rats (250-300 g) were used in the study (n = 36).

The rats were divided into three groups: Control group consisted of rats which were anesthetized with ketamine (n = 12); in ischemia group, the rats were reperfused for 4 hours following ischemia for 6 hours (n = 12) and, in clopidogrel-pretreated group, the rats were pretreated with clopidogrel (Plavix, Sanofi-Synthelabo, Istanbul, Turkey; 0.2 mg/kg/day) for 10 days prior to ischemia-reperfusion (n = 12). Clopidogrel was administered to the animals via a nasogastric tube.

On the 11th day, circulation of right lower extremity of the animals was blocked at trochanter major level. Blockade of circulation was confirmed with Doppler ultrasonography (MD 2, Huntleigh Diagnostics Ltd., South Glamorgan, UK). This method has been shown previously to reduce the blood flow approximately 98% and lead to acute ischemia (Mathru et al. 1996). After 6 h of ischemia, the extremity was reperfused for 4 h. Then, all rats were anesthetized with ketamine (100 mg/kg; Ketalar, Pfizer, Istanbul, Turkey). Blood samples (5 ml) were drawn from the heart and ascending aorta following midsternotomy. Tissue samples were taken from gastrocnemius muscle, liver, and lungs. Malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) levels were measured in blood and tissue samples.

Biochemical assays

Tissues were washed in 0.9% NaCl and homogenized with cold Tris/HCl buffer (pH 7.4). Tissue MDA levels -end product of lipid peroxidation- were determined according to Buege and Aust method (Buege and Aust 1978). The results were expressed as nmol/mg protein. Tissue GSH was measured spectrophotometrically according to Ellman procedure (Ellman 1959) and the results were expressed as μmol/mg protein. Protein concentrations of tissue homogenates were determined by Lowry protein assay (Lowry et al. 1951). Cu-ZnSOD activity was measured kinetically by a method described by Sun et al. (1988). Plasma samples were used for the measurement of MDA, and red blood cell suspensions were used for the measurement of GSH and SOD levels.

Statistical analysis

All data are expressed as means ± S.D. and analyzed by Kruskal Wallis test. For the comparison of paired data, Mann Whitney's U-test was used. Values of $p < 0.05$ were regarded as significant. Calculations were done using SPSS Version 11.5 software package.

Results

MDA Levels

Ischemia-reperfusion increased MDA levels in plasma and all tissues except for the lungs. Kruskal Wallis test revealed significant differences between ischemia-reperfusion and control groups for liver ($p = 0.003$), muscle ($p < 0.001$) and plasma ($p = 0.008$) (Table 1). Clopidogrel pretreatment prevented ischemia-reperfusion-induced increases in MDA levels in liver, muscle and plasma. Comparison between ischemia and clopidogrel-pretreated groups showed significant differences for liver, muscle and plasma ($p = 0.003$, $p = 0.001$ and $p = 0.029$, respectively) (Table 1).

GSH Levels

Unlike MDA levels, ischemia-reperfusion caused significant decreases in GSH levels in all tissues including the red blood cells. Comparison between control and ischemia groups revealed significant differences as $p = 0.006$, $p = 0.006$, $p = 0.002$ and $p = 0.001$ for hepatic, lung, muscle and red blood cell GSH levels, respectively (Table 2). Clopidogrel pretreatment prevented the decrease in GSH levels in muscle ($p = 0.003$) and red blood cells ($p < 0.001$), but not those of lungs and liver compared to ischemia (Table 2). There was no difference between the GSH levels of control and clopidogrel-pretreated groups.

SOD Activity

Hepatic, lung, muscle and red blood cell SOD activities showed significant decreases due to ischemia-reperfusion when compared with controls ($p = 0.001$, $p = 0.001$, $p = 0.006$ and $p = 0.001$, respectively). Clopidogrel pretreatment prevented ischemia-reperfusion-induced decreases in SOD activity in all tissues including the red blood cells compared to ischemia group ($p = 0.001$ for liver, lungs and red blood cells, and $p = 0.008$ for muscle). There was no difference between the SOD levels of control and clopidogrel-pretreated groups (Table 3).

Table 1. *Tissue and blood MDA levels (nmol/100 mg protein) in control, ischemia and clopidogrel-pretreated ischemia groups*

Tissue		Control ($n = 12$)	Ischemia ($n = 12$)	Clopidogrel-pretreated ischemia ($n = 12$)
Liver	Mean ± S.D.	60.3 ± 17.5	104.8 ± 37.5	39.2 ± 18.4
	Median	58.0	94.9 [a]	45.5 [e]
Lung	Mean ± S.D.	90.9 ± 25.7	190.8 ± 101.6	90.2 ± 30.7
	Median	109.9	195.6 [b]	99.6 [f]
Muscle	Mean ± S.D.	37.6 ± 4.7	73.7 ± 30.5	39.1 ± 6.8
	Median	40.0	58.9 [c]	33.7 [g]
Plasma	Mean ± S.D.	10.8 ± 8.2	130.2 ± 86.5	38.2 ± 42.2
	Median	11.0	141.0 [d]	18.1 [h]

No significant difference was detected between control and clopidogrel- pretreated groups.
[a]$p = 0.025$, [b]$p = 0.079$, [c]$p = 0.005$, [d]$p = 0.008$: Control vs Ischemia.
[e]$p = 0.003$, [f]$p = 0.110$, [g]$p = 0.001$, [h]$p = 0.029$: Clopidogrel-pretreated ischemia vs Ischemia.

TABLE 2. *GSH values of tissues (nmol/100 mg protein) and red blood cells (mg/100 ml) in control, ischemia and clopidogrel-pretreated ischemia groups*

Tissue		Control (n = 12)	Ischemia (n = 12)	Clopidogrel-pretreated ischemia (n = 12)
Liver	Mean ± S.D.	19.8 ± 3.1	16.2 ± 5.9	21.9 ± 8.2
	Median	19.7	14.9 [a]	17.8 [e]
Lung	Mean ± S.D.	27.2 ± 11.3	10.9 ± 2.9	18.0 ± 5.7
	Median	19.9	11.0 [b]	13.9 [f]
Muscle	Mean ± S.D.	76.5 ± 7.0	33.7 ± 16.4	81.0 ± 38.4
	Median	78.9	29.1 [c]	59.2 [g]
Red blood cell	Mean ± S.D.	51.9 ± 8.4	31.3 ± 7.3	57.2 ± 17.7
	Median	50.0	33.1 [d]	51.0 [h]

No significant difference was detected between control and clopidogrel-pretreated groups.
[a]$p = 0.006$, [b]$p = 0.006$, [c]$p = 0.002$, [d]$p = 0.001$: Control vs Ischemia.
[e]$p = 0.062$, [f]$p = 0.051$, [g]$p = 0.003$, [h]$p < 0.001$: Clopidogrel-pretreated ischemia vs Ischemia.

TABLE 3. *SOD activities in tissues (U/mg protein) and red blood cells (U/100 mg Hb) of control, ischemia and clopidogrel-pretreated ischemia groups*

Tissue		Control (n = 12)	Ischemia (n = 12)	Clopidogrel-pretreated ischemia (n = 12)
Liver	Mean ± S.D.	25.4 ± 9.2	11.6 ± 2.5	23.2 ± 5.1
	Median	24.1	11.6 [a]	22.6[e]
Lung	Mean ± S.D.	21.5 ± 3.6	10.8 ± 2.4	21.0 ± 4.5
	Median	22.3	11.1 [b]	20.9 [f]
Muscle	Mean ± S.D.	37.7 ± 2.3	18.0 ± 8.9	35.9 ± 19.7
	Median	35.9	19.0 [c]	23.6 [g]
Red blood cell	Mean ± S.D.	92.4 ± 15.3	58.6 ± 8.9	92.2 ± 14.5
	Median	105.8	54.5 [d]	97.2 [h]

No significant difference was detected between control and clopidogrel-pretreated groups.
[a]$p = 0.001$, [b]$p = 0.001$, [c]$p = 0.006$, [d]$p = 0.001$: Control vs Ischemia.
[e]$p = 0.001$, [f]$p = 0.001$, [g]$p = 0.008$, [h]$p = 0.001$: Clopidogrel-pretreated ischemia vs Ischemia.

DISCUSSION

Following acute lower extremity ischemia, death and amputation incidences are reported to be 15-52% and 12-22%, respectively. Complete healing rate is only 60-70% with the most effective therapy (Homer-Vanniasinkam and Gough 1994). The main complication of the lower extremity ischemia is "myonephropathic metabolic syndrome." During ischemia, endothelial and leukocyte functions impair due to several factors triggered by platelet activation (Dunzendorfer et

al. 2002). Neutrophil or platelet aggregation, vasoconstriction, increase in capillary permeability and structural endothelial changes are the major changes that occur during ischemia (Braithwiate et al. 1995).

Reperfusion of an extremity following an extended period of ischemia causes the release of free oxygen radicals. Intracellular pH shifts towards acidity due to anaerobic metabolism, leading to impaired functions of cellular membranes during ischemia. This results in the exchange of calcium and other cations and the reduction of adenosine triphosphate to inosine and hypoxanthine (Ihnken et al. 1996). The changes in the intracellular calcium level and the presence of hypoxanthine lead to the production of free oxygen radicals during the reperfusion period. Then, the oxidants bind to fatty acids of the cell membranes and initiate lipid peroxidation (Ikezawa et al.1993; Beyersdof 1995). They also interact with the endothelial surfaces and neutrophils, which causes a further increase in lipid peroxidation. Cellular swelling, edema, increase in myoglobin and oxidants lead to renal, pulmonary, and cerebral dysfunction (Groeneveld et al. 1997). Loss of extremity viability or even death may be inevitable (Lu et al. 1997). Therefore, early diagnosis and early treatment during reperfusion are vital to prevent irreversible consequences.

The organism protects itself from oxidative stress by endogenous free radical scavenger systems. One of the endogenous antioxidant enzymes is SOD. SOD eliminates the superoxide radicals by converting them to H_2O_2, which is reduced to water by cytosolic antioxidants, catalase and glutathione peroxidase (Ferrari et al. 1991). GSH is another key factor involved in the detoxification of electrophilic metabolites and reactive oxygen intermediates (Ferrari et al. 1986). GSH, as a co-substrate of glutathione peroxidase, plays an essential role in protection from free radicals (Curello et al. 1985).

In the present study, it is observed that ischemia-reperfusion caused free radical production (as shown in increased MDA levels) with a compensatory decrease in GSH levels and SOD activity. Pretreatment with clopidogrel prevented these changes. The antiplatelet agent, clopidogrel is a thienopyridine derivative. It inhibits platelet ADP receptor, P2T, resulting in inhibition of platelet activation and aggregation. This antagonism is non-competitive, irreversible, and results in 50-70% inhibition of platelet fibrinogen binding. Clopidogrel has also been reported to prevent thrombocyte aggregation by inhibiting fibrinogen binding to activated fibrinogen receptors (GPllb-llla complex) on the platelets (Dunn et al. 1984; Di Minno et al. 1985). Inhibition of platelet aggregation occurs 24-48 h following the oral intake of clopidogrel and reaches its maximum level in 3 to 5 days. Restoration of platelet functions occurs slowly within 7-14 days, after withdrawal of the drug.

It has been shown that clopidogrel increases the perfusion of tissues by decreasing platelet and leukocyte adhesion and reducing blood viscosity in cases with peripheral artery diseases (Pasqualini et al. 2002). Platelet aggregation due to increased permeability in major organs during reperfusion was reported to be prevented by clopidogrel (Jeffrey et al. 1999). Thus, the inhibition of platelet aggregation by preventing endothelial and leukocyte dysfunction might be one of the mechanisms of the to drug to prevent the reperfusion injury (Dunzendorfer et al. 2002).

It has been demonstrated that treatment with clopidogrel decreases chemokinesis of monocytes. Pretreatment of endothelial cells with clopidogrel has been shown to decrease the priming of the endothelial cells by tumor necrosis factor and neutrophil transmigration (Dunzendorfer et al. 2002). Therefore, the decrease in monocyte chemokinesis and TNF level by the drug may protect the tissues during the reperfusion period.

In conclusion, although the exact mechanism of ischemia-reperfusion injury has not been clearly understood yet, the results of this study demonstrate that clopidogrel decreases reperfusion injury via inhibition of free radical production. Therefore, this agent, which is currently being used for the treatment of atherosclerosis, might also be useful in the prevention of reperfusion injuries.

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