Biomed. Papers 145(2), 69–74 (2001)

APPLICATION OF CATIONIC PROPYL GALLATE AS INDUCER OF THROMBOCYTE AGGREGATION FOR EVALUATION OF EFFECTIVENESS OF ANTIAGGREGATION THERAPY

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Received: January 10, 2002

Key words: Cationic propylgallate / Platelet aggregation / Thrombocyte aggregation / Acetylsalicylic acid

Introduction and aim of the study: Acetylsalicylic acid (ASA) is one of basic preparations used in the therapy of cardiovascular diseases. Application of ASA leads to irreversible reduction of platelet aggregation. The aim of the present study was to verify monitoring of effectiveness of ASA therapy using the measurement of platelet aggregability in vitro after induction by cationic propyl gallate (CPG), which is considered to be a highly potent inducer of aggregation.

Methods: We examined a group of 27 healthy volunteers, divided into two subgroups (n = 19, n = 8). The first subgroup was examined for thrombocyte aggregation before and 24 hours after administration of 400 mg of ASA after induction by ADP, collagen, adrenalin and CPG. The second subgroup was examined for thrombocyte aggregation before and after a three-day administration of ASA in a dose of 100 mg/day.

Results and conclusion: In the group of 27 healthy volunteers we determined normal values of aggregability for individual inducers. Low stability of the used methods was proved (weak or insignificant correlation of results of the same method before and after administration of ASA). The most advantageous parameter for monitoring of effectiveness of 400 and 100 mg of ASA was CPG slope (paired t test, $p < 0.00000002$, resp. $p < 0.001$). The parameter of CPG slope we determined in both subgroups the cut-off value (<53s), by means of which it is possible to discriminate probands according to ASA therapy (in contrast to other routinely used inducers).

The obtained results indicate that measurement of thrombocyte aggregation after CPG induction reveals a significantly lower percentage of ASA non-responders ASA than after other inducers.

Measurement of thrombocyte aggregation after CPG induction is predicted to be highly promising for monitoring the effectiveness of anti-aggregation therapy.

INTRODUCTION

Acetylsalicylic acid (ASA) is a basic drug and has been used particularly in prevention of myocardial infarction and other cardiovascular diseases. It blocks cyclooxygenase, which plays a crucial role in thromboxane A2 synthesis – this results in irreversible reduction of platelet aggregation during their lifetime. Recently, much data have been published effect of ASA. Some authors showing 30% inadequate responders[1, 6, 8].

To date, the effectiveness of ASA therapy had been monitored by examination of thrombocyte aggregability in vitro using common aggregation inducers (ADP, collagen, adrenalin, arachidonic acid, etc.). However, these methods have low sensitivity, specificity and reproducibility.

A new method used for monitoring of effectiveness of ASA therapy is measurement of thrombocyte aggregation after induction by cationic propyl gallate (CPG).

Propyl gallate is alkyl ester of gallic acid. It has strong antioxidative effects, blocks activity of lipooxygenase, stimulates the releasing reaction from platelet granules, modifies composition of blood platelet membrane and induces binding of annexin[9, 10, 11]. Aggregation of thrombocytes after CPG induction is blocked by ASA (inhibitor of cyclooxygenase)[12]. When using CPG, 100% sensitivity was reported in detection of impaired aggregability caused by ASA[12].

The aim of the present study was as follows:

1. assessment of the effect of various doses of ASA (100 mg/day, 400 mg/day) on change of aggregation curve after aggregation induced by adrenalin, ADP, collagen and CPG;
2. determination of cut-off values of aggregability with the above mentioned aggregation inducers after therapy by various doses of ASA;
3. comparison of sensitivity of individual aggregation inducers to assess efficiency of ASA therapy.

METHODS

Group of examined persons

The study comprised 27 healthy volunteers of mean age of 33.6 years, who during the last 14 days had not used ASA or other drugs affecting platelet aggregability; the day before examination they were not allowed to smoke or consume alcohol; sample collection was performed in morning hours on fasting.

The whole group was divided by the method of random selection into two subgroups; the first (n = 18) for followed up for thrombocyte aggregability after induction by ADP, collagen, CPG and adrenalin before and 24 hours after administration of 400 mg of ASA (Anopyrin 400, group ASA400). They were probands on whom we tested the effect of one higher dose of ASA.

The second group (n = 9) was followed up for aggregability before and 3 days after administration of 100 mg of ASA (Anopyrin 100, group ASA100). They were probands on whom we tested the cumulative effect of several lower doses of ASA.

Isolation of platelets and sample collection

Samples of venous blood were collected under standard conditions: in the morning, after 12 hour fasting, from non-retracted arm, by a thick transfusion needle. Prior to each sample collection, the first sample (15 ml of blood) was poured off. We then collected 4.5 ml of blood into a plastic test tube containing 0.5 ml of 3.8% Na citricum (for analysis of aggregation response after induction we used citrate plasma – 0.106 mol/l of Na citricum, ratio of Na citrate and blood 1:9).

Samples were processed within one hour.

Platelet-rich plasma was prepared by centrifugation (PRP – containing 200 000 – 400 000 platelets/μl, centrifugation 700–800 rpm for 10 min), as well as platelet-poor plasma (PPP – containing < 7000 platelets/μl, centrifugation 3000 rpm for 20 min).

Measurement of platelet aggregability

Platelet aggregation was determined by means of the Born turbidimetric method (optical aggregometer APACT II, Labor Fibrintimer) after addition of aggregation inducers (100 umol/l ADP, 110 mg/ml of collagen, 550 umol/l of adrenalin, and 300 umol/l of CPG).

From the aggregation curve we evaluated % aggregation, slope (% aggregation per minute), duration of lag phase when using collagen and T 50 (time necessary for reaching 50 % of total aggregation) with CPG.

Statistical analysis

The data were processed by statistical analysis (software SPSS). To determine significance of differences between the measured values before and after therapy, we used pair Student's t-tests. For evaluation we also used Pears correlation coefficient and nonparametric U tests. As data distribution in all parameters before therapy was almost normal, for assessment of normal values we used the mean and standard deviation (x + –2sd); for assessment of cut-off values we used the limit values (minimum, maximum).

RESULTS

We examined the group of 27 healthy volunteers, mean age 33.6 years (25–59 years) in whom normal values of aggregation parameters for individual inducers were determined (Table 1).

During determination of relations among individual parameters measured (after induction by various inducers) prior to ASA therapy, we found numerous significant linear relations (Table 2).

Measurement of aggregation response after a single administration of 400 mg of ASA to 18 probands showed no correlation before and after application of the same method. Significant correlations were found only in CPG and collagen (Table 1).

After therapy, the statistically significant changes were recorded in most inducers; the most significant were in T50CPG and slope CPG (Table 1).

After a single administration of 400 mg of ASA, we found many relations between aggregations and individual inducers (Table 2).

In over a half of probands ASA400, after ADP induction disaggregation was found (disintegration of the originated thrombocyte aggregate); application of other inducers resulted in minimal disaggregation. Therefore we divided the group of 18 probands into subgroups according to the presence (absence) of disaggregation. The groups were compared by means of nonparametric U tests; no significant difference was found in any aggregation parameter, namely no correlation was found between disaggregation and aggregation.

Determination of cut-off values for single administration of 400 mg of ASA revealed that the limit values could be assessed reliably in parameters of slope CPG (<53 s) and T50CPG (>91s).

In the group ASA100, most probands did not show any significant relations among the parameters measured before and after administration of ASA (significant correlation was found only in the lag phase of collagen) (Table 3).

Moreover, this group showed no significant changes of aggregation by most inducers after the therapy (in contrast to ASA400); statistically significant changes were recorded only with collagen and CPG; the most significant was the change in slope CPG (Table 3).

After a three-day administration of 100 mg of ASA we found less significant correlations among individual inducers than after a single administration of 400 mg of ASA (Table 4).

Determination of cut-off values for 100 mg of ASA showed that limit values could be assessed reliably from the parameter slope CPG (<53s).

Tables 5 and 6 indicate high sensitivity of the test of aggregation with CPG at detection of platelet inhibition caused by 100 and 400 mg of ASA. After induction by CPG we recorded significantly changed values of the measured parameters in most probands. These probands also showed a high percentage of negative results when using other aggregation inducers – 30% in ADP at the dose of 100 and of 400 mg/d of ASA, 60% in collagen at the dose of 100mg/d of ASA. Only one person from the whole group (100 mg of ASA) did not show any significant changes in aggregation parameters after induction by CPG (insufficient reaction to induction by collagen and ADP was proved as well).

Table 1. Basic statistics, 17 probands, 400 mg of ASA

Parameter	X	S	"Normal value"		Median	Minimum	Maximum	10. perc	90. perc.	Cor	R
Age	33.60	9.40	14.80	52.40	31.00	25.00	59.00				
ADP%_I	72.10	9.20	53.70	90.50	72.00	58.40	86.10	58.90	84.90		
ADP%_II	57.70	12.10	33.50	81.90	58.90	30.80	73.70	46.20	71.10	NS	VS (0.003)
ADPSl_I	83.70	11.20	61.30	106.10	80.40	67.90	108.70	74.50	100.00		
ADPSl_II	80.60	19.20	42.20	119.00	82.30	40.40	112.30	59.30	103.60	NS	NS
Coll%_I	71.60	7.80	56.00	87.20	72.30	57.70	90.20	62.80	80.00		
Coll%_II	43.20	28.70	-14.20	100.60	43.50	8.30	80.30	8.70	79.60	NS	VS (0.001)
CollSl_I	79.40	14.70	50.00	108.80	82.80	41.40	100.40	61.00	96.10		
CollSl_II	38.20	27.20	-16.20	92.60	41.30	5.80	73.40	5.90	73.10	S (kk 0.52)	VS (0.000005)
CollLag_I	36.10	12.60	10.90	61.30	33.00	15.00	62.00	24.00	56.00		
CollLag_II	54.80	29.40	-4.00	113.60	40.50	20.00	110.00	26.00	102.00	VS (kk 0.82)	VS (0.005)
Adren%_I	73.70	9.00	55.70	91.70	72.50	60.70	90.10	65.20	85.20		
Adren%_II	25.50	8.30	8.90	42.10	24.30	12.00	38.80	17.00	34.00	NS	S (0.02)
AdrenSl_I	38.80	15.20	8.40	69.20	35.00	24.30	74.40	26.80	52.40		
AdrenSl_II	25.50	8.30	8.90	42.10	24.30	12.00	38.80	15.70	22.90	NS	S (0.016)
PG%_I	75.80	8.80	58.20	93.40	75.00	60.70	93.60	63.10	91.10		
PG%_II	69.90	15.60	38.70	101.10	70.20	33.20	100.00	51.90	97.10	NS	NS
PGSl_I	78.30	12.80	52.70	103.90	77.80	56.70	102.70	60.80	96.20		
PGSl_II	36.30	9.50	17.30	55.30	37.10	17.70	52.60	25.00	50.30	NS	VS (0.0000002)
T50PG_I	68.90	12.60	43.70	94.10	68.90	55.00	106.00	56.00	81.00		
T50PG_II	122.20	32.40	57.40	187.00	114.00	85.00	198.00	91.00	175.00	S (kk 0.55)	VS (0.0000002)

X..mean S..standard deviation Cor…….. Significance of ev.correlation between the same parameter before and after therapy

R..significant difference before and after therapy for each parameter (significance of pair test) NS..p>0.05 S..p<0.05 VS..p<0.01

X_I..before therapy X_II..after therapy

X%..%aggregation after induction X XSI..slope after induction X Xlag..lag phase after induction X T50X..time necessary to reach 50% aggregation after induction X

Table 2. Significant correlation before ASA application

Parameter	very strong	strong	mean
ADP%-I		CollLag(-0.68). ADPSL-I(0.6)	CollSl-I (0.55)
ADPSl-I		Coll-I(0.65). ADP%-I (0.6). CollSl-I (0.5)	
Coll%-I		AdreSl-I (0.75). CollSl-I(0.74). ADP%-I(0.67). ADPSl-I (0.65)	T50PG (-0.58). CollLag (-0.56).
CollSl-I		Adren%-I (0.8). Coll-I (0.74). AdreSl-I (0.71). T50PG (-0.71). Colllag-I(-0.69)	ADP%-I (0.55). ADPSl-I (0.5).
CollLag-I		Adr%-I (-0.71). ADP%-I (-0.68). CollSl (-0.69)	Coll%-I (-0.56).
Adr%-I		CollSl-I (0.8). AdrSl-I (0.73). CollLag-I (-0.71)	
AdrSlo-I		Coll%-I (0.75). Adr%-I (0.73). CollSlo-I (0.71)	
PG%-I		CollSl (0.62)	PGSlo-I (0.56). CollLag (-0.52).
PGSlo-I			PG%-I (0.56)
T50PG-I		CollSl-I (-0.71)	Coll%-I (-0.58).

Significant correlation after ASA application.

Parameter	very strong	strong	mean
ADP%-II	ADPSl-II (0.93).PGSl-II (0.81)	PG%-II (0.71). Coll%-II (0.69). CollSl-II (0.69)	CollLag-II(-0.56)
ADPSl-II	ADP%-II (0.93)	Coll%-II (0.66). CollSl-II (0.67). Colllag-II (-0.65). AdrSl-II (0.68. PGSl-II (0.62)	PG%-II (0.54).
Coll%-II	CollSl-II (0.99). Colllag-II (-0.85	ADP%-II(0.69). ADPSl-II (0.66).	PG%-II (0.52). PGSlo-II (0.52)
CollSl-II	Coll%-II (0.99). Colllag-II (-0.86	ADP%-II (0.69. ADPSl-II (0.67).	
Colllag-II	CollSl(-0.86). Coll%-II (-0.85.	ADPSl-II (-0.65)	ADP%-II (-0.56)
Adr%-II		AdrSlo-II (0.77)	
AdrSl-II		Adr% (0.77). ADPSl-II (0.68)	
PG%-II	PGSl-II (0.9)	ADP%-II (0.71)	ADPSl-II (0.54). Coll%-II (0.52)
PGSl-II	PG%-II (0.9). ADP%-II (0.81)	ADPSL-II(0.62)	Coll%-II (0.52)
T50PG-II			

Table 3. Basic statistics, 9 probands, 100 mg of ASA

Parameter	X	S	"Normal value"		Median	Minimum	Maximum	Cor	R
Age	28.70	7.10	14.50	42.90	28.00				
ADP%_I	68.20	6.10	56.00	80.40	69.00	58.40	75.30		
ADP%_II	57.80	17.20	23.40	92.20	62.50	35.00	78.90	NS	NS
ADPSl_I	80.00	8.70	62.60	97.40	77.90	73.70	98.40		
ADPSl_II	81.30	14.20	52.90	109.70	80.00	65.00	100.60	NS	NS
Coll%_I	84.70	6.60	71.50	97.90	72.80	66.20	89.00		
Coll%_II	76.50	5.50	65.50	87.50	77.10	68.90	84.70	NS	NS
CollSl_I	92.00	19.50	53.00	131.00	90.60	65.50	132.20		
CollSl_II	66.30	5.10	56.10	76.50	64.40	60.40	75.30	NS	S (0.01)
CollLag_I	36.30	17.80	0.70	71.90	37.50	15.60	72.00		
CollLag_II	25.70	12.60	0.50	50.90	21.70	12.80	52.00	S (kk 0.83)	S (0.02)
PG%_I	77.20	9.00	59.20	95.20	75.20	65.30	93.60		
PG%_II	72.80	6.20	60.40	85.20	72.80	65.40	85.20	NS	NS
PGSl_I	79.40	12.90	53.60	105.20	81.30	60.80	96.60		
PGSl_II	37.50	7.70	22.00	53.00	37.90	29.50	56.90	NS	VS (0.001)
T50PG_I	68.40	12.60	43.20	93.60	65.00	52.00	88.00		
T50PG_II	126.10	65.10	-4.10	256.30	108.60	65.40	272.00	NS	S (0.03)

X..mean S..standard deviation Cor…….. Significance of ev.correlation between the same parameter before and after therapy

R..significant difference before and after therapy for each parameter (significance of pair test) NS..p>0.05 S..p<0.05 VS..p<0.01

X_I..before therapy X_II..after therapy

X%..%aggregation after induction X XSI..slope after induction X Xlag..lag phase after induction X T50X..time necessary to reach 50% aggregation after induction X

Table 4. Significant correlation before ASA application

Parameter	very strong	strong
ADP%-I		T50PG-I (0.77). Coll%-I(0.76)
Coll%-I	CollSl-I (0.89)	ADP%-I (0.76)
CollSl-I	Coll%-I (0.89)	CollLag-I (-0.8)
CollLag-I		CollSl-I (-0.8)
PG%-I		CollSl (0.62)
T50PG-I		ADP%-I (0.76)

Significant correlation after ASA application.

Parameter	very strong	strong
ADPSl-II		ADP%-II (0.77)
ADP%-II		ADPSl-II (0.77)
Colllag-II	T50PG-II(0.83)	ADPSl-II (-0.65)
PG%-II		PGSl-II (0.71)
PGSl-II		PG%-II (0.71)
T50PG-II	Colllag-II (0.83)	

Table 5. Negative results after application of 400 mg of ASA. Comparison of common aggregation inducers with CPG

Inducer	No. of ineffective	Out of them	SL CPG effective	T50CPG effective
Collagen	5 (30%)		5 (100%)	5 (100%)
ADP	2 (11%)		2 (100%)	2 (100%)
Adrenalin	2 (11%)		2 (100%)	2 (100%)

Table 6. Negative results after repeated application of 100 mg of ASA. Comparison of common aggregation inducers with CPG

Inducer	No. of ineffective	Out of them	SL CPG effective	T50CPG effective
Collagen	6 (60%)		5 (84%)	5 (84%)
ADP	2 (20%)		2 (100%)	1 (50%)
Adrenalin	2 (20%)		2 (100%)	2 (100%)

DISCUSSION

The literature reports several methods for monitoring the efficiency of ASA therapy.

This was based on the fact that ASA has been used as a basic drug for secondary prevention of ischemic disease of the heart and brain. The literature however describes 30% of non responders to ASA therapy[1, 6, 8]. With respect to a low reliability of the used laboratory techniques, evaluation of non responders could be error-loaded[12].

The most common determination of efficiency of ASA therapy for assessment of aggregability of thrombocytes in vitro is after induction by thrombin, collagen, adrenalin, arachidonic acid and ADP. However, these methods have low sensitivity, specificity and reproducibility[12].

Another method used for the assessment of effectiveness of ASA therapy is the measurement of thromboxan B2 (TXB2) – a stable metabolite of thromboxan A2 (TXA2)[2]. In this context it is highly interesting that some probands do not show any efficient reduction of platelet aggregation after stimulation by common inducers even after decreased TXB2 (i. e. at signs of efficient blockage of cyklooxygenase)[5, 7].

This may be explained by the variability of the grade of inhibition of TXA2 by acetylsalicylic acid, relative importance of platelet aggregability dependent on extra-arachidonic pathways, and mainly by unreliability of present aggregation tests using common aggregation inducers[5, 12, 13].

Significant linear correlations among individual parameters (after application of individual inducers) found in the group ASA400 were expected.

No correlation among the results of the same method before and after administration of 400 mg of ASA is interpreted as a low stability of the used methods; our findings correspond to literary data[12].

As the most statistically significant changes after the therapy occurred in T50CPG and slope CPG, we consider these parameters as the most favorable for monitoring efficiency of the therapy by a daily dose of 400 mg of ASA. Our findings correspond to literary data[12]. Significance changes of aggregation parameters after induction by adrenalin was also high, but on the basis of our experience, mainly due to very bad reproducibility of examinations, the use of adrenalin is not recommended.

The fact that the group ASA100 did not show any significant aggregation changes after therapy using most inducers is considered by us as evidence of low sensitivity of most methods used – on the contrary, the finding of statistically significant changes in CPG indicates a higher reliability of this parameter for monitoring efficiency of the therapy using a daily dose of 100 mg of ASA.

One proband from the whole group (6%) we classified as an ASA non-responder (no reaction of aggregation was proved to ASA therapy after CPG induction; no reaction to ADP, collagen and adrenalin was recorded).

The literature reports a significantly higher percentage of ASA non-responders[5, 12, 13]; with regard to a low reliability of the commonly used methods (inducers) and low correlation between TXB2 values and thrombocyte aggregation (after stimulation by common inducers) we assume that the real occurrence of non-responders is lower. This is based, apart from literary data, on the proved false negativity (insufficient aggregation) in the commonly used inducers compared to CPG[12].

CONCLUSION

We examined aggregation responses to induction by various inducers in a group of 27 healthy volunteers and determined normal values for individual parameters of aggregation.

Then we selected parameters with the highest sensitivity to follow up efficiency of ASA administration. We also succeeded in determining the cut-off values for daily doses of 100 mg and 400 mg of ASA (induction by cationic propyl gallate – CPG).

The results obtained indicate that the measurement of thrombocyte aggregation after CPG induction give a significantly lower percentage of ASA non responders than after other inducers.

We believe that measurement of thrombocyte aggregation after CPG induction is highly promising for monitoring the efficiency of antiaggregation therapy.

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