

Cross talk between oxidative stress, PPAR- γ and RAS system in diabetic rat heart exposed to ischemia / reperfusion injury

Abstract

Introduction: The present study aimed at clarifying the cross talk between PPAR- γ , cardiac ROS and RAS. **Methods:** A total of 90 male albino rats were used. They rats were divided into: *Group 1:* Control group, *Group 2:* Type 2 diabetic rats, *Group 3:* PPAR γ agonist protected type 2 diabetic rats. *Group 4:* Antioxidant protected type2 diabetic rats, *Group 5:* Metformin treated type 2 diabetic rats. Blood samples were collected for measurement of FBS and fasting insulin. Half the number of each group was sacrificed and the heart excised and perfused, from the rest of the group small piece from the heart was taken for estimation of MDA, AT2R and ACE2 gene expression. **Results:** Treatment with pioglitazone and Vitamin E significantly lowered blood glucose, insulin levels and HOMA IR. However, values did not return to control values. Pioglitazone and Vitamin E improved myocardial performance and percentage recovery following ischemia reperfusion. The cardioprotective effect was more pronounced in the pioglitazone group. This positively correlated with decreased MDA levels and

20 increased AT2R and ACE2 expression in cardiac tissue. **In Conclusion,**
21 Pioglitazone and Vitamin E in type 2 DM significantly offered cardio-protection
22 through improving the diabetic condition and / or decreasing MDA levels.

23 **Key words:** *Type 2 Diabetes mellitus, cardiac ischemia reperfusion, PPAR γ ,*
24 *oxidative stress, ACE2, AT2R expression.*

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26 **Introduction and aim of the work**

27 Diabetes mellitus (DM) leads to high cardiovascular morbidity and mortality as a
28 result of functional and morphological damage in diabetic hearts.

29 Most of the diabetes-related cardiovascular complications can be largely
30 attributed to overproduction of reactive oxygen species such as superoxide and
31 hydrogen peroxide, and/or depletion of antioxidant defense mechanisms [1].

32 Antioxidant therapy has shown promise in prevention of development of
33 diabetic heart complications but the results of intervention studies with classic
34 antioxidants such as vitamin E, were shown to be elusive and unsuccessful [2]. On
35 this matter, it has recently been suggested that antioxidant therapy with vitamin E
36 or other antioxidants is limited to scavenging already-formed oxidants and may
37 therefore be considered a more "symptomatic" rather than a causal treatment for

38 vascular oxidative stress. Hyperglycemia-induced oxidative stress is accompanied
39 by increased production of advanced glycosylation end products (AGEs),
40 upregulation of the receptor for AGEs (RAGE) [3] and downregulation of
41 peroxisome proliferator-activated receptor-gamma (PPAR γ) system [4].

42 AGE binds to its cell surface receptor, RAGE, resulting in the activation of
43 postreceptor signaling, generation of intracellular reactive oxygen species, and
44 alteration of gene expression[5]

45 Reactive oxygen species have been indicated as one of the earliest and most
46 important components of tissue injury after reperfusion of ischemic organ. Free
47 radical production is continuous during ischemia, while during reperfusion it is
48 primarily confined to the early stage when fresh oxygen is supplied to the ischemic
49 region [6].

50 PPAR- γ system plays a critical role in oxidative stress that seems to be
51 implicated in the development of insulin resistance and diabetic complications.
52 Moreover, current evidence points to a cardioprotective role for PPAR- γ ligands in
53 ischemia reperfusion animal models, but the functional role of PPAR- γ in diabetic
54 hearts is not fully understood [7].

55 On the other hand, it has been recently suggested that angiotensin signaling may
56 be regulated at multiple levels by PPAR- γ ligands, significantly reducing the

57 expression of AT1 receptor and repressing signal transduction through this receptor
58 to suppress oxidative stress. However, the relation between PPAR- γ , MDA levels,
59 AT2 receptor and ACE2 expression in diabetic hearts needs to be clarified [8].

60 **The aim of this study is to:**

- 61 1. Test PPAR- γ agonist and antioxidant therapy on the development of type 2
62 DM in rats.
- 63 2. Test the possibility of improving the myocardial function and reducing the
64 susceptibility to ischemia reperfusion injury through reducing MDA, one of
65 the biomarkers of the oxidative stress.
- 66 3. Estimate the cardiac ACE2 and AT2R gene expression in relation to PPAR-
67 γ system.
- 68 4. Compare the possible cardio-protective effects of PPAR- γ agonist,
69 antioxidant therapy and metformin in type 2 diabetic hearts.

70 **Materials and Methods**

71 ***Experimental Animals:***

72 A total of 90 male albino rats were used in this study weighing 160-180
73 grams. The animals were housed in animal house of faculty of Medicine, Cairo

74 University, in wire mesh cages at room temperature, with 12 hour light dark cycle.

75 They were fed the commercial rat chew diet and had free access to water.

76 These rats were divided into the following groups:

77 **Group 1: Control group**

78 Consists of 18 normal rats.

79 **Group 2: Type 2 diabetic rats.**

80 Type 2 diabetic rats (n=18).

81 **Group 3: PPAR γ agonist protected type2 diabetic rats group**

82 Rats was treated with oral pioglitazone 20 mg/kg/day for the whole time of the
83 experiment (n=18).

84 **Group 4: Antioxidant protected type2 diabetic rats group**

85 Rats was treated with oral vit E 500 mg/kg/day for the whole time of the
86 experiment (n=18).

87 **Group 5: Metformin treated type2 diabetic rats group**

88 Treatment was initiated at 350 mg/kg/ day and was gradually increased to a
89 dose of 650 mg/kg/ day for the whole time of the experiment (n=18).

90 Half the number of each group was sacrificed and the heart excised and
91 perfused according to the Langendorff technique, from the rest of the group small

piece from the heart was taken for estimation of AT2R and ACE2 expression and malondialdehyde (MDA).

Experimental protocol:

Induction of Type 2 Diabetes:

Beginning on day 0, rats were fed either normal rodent chow (12% calories as fat)(group 1) or high fat diet (60% calories as fat)(group 2) for two weeks. On day 14, rats on the high fat diet (HFD) were injected with a single low dose of streptozotocin (STZ, 35 mg/kg i.p., in 0.01M citrate buffer pH 4.3) to induce type 2 diabetes [9]. Consumption of high fat diet leads to insulin resistance and is considered to be a major predisposing factor for type 2 diabetes [10]. Subsequent to STZ treatment, rats had free access to food and water and were continued on their respective diets for the duration of study.

Characterization of Type 2 Diabetes Model

On day 20, in the HFD + STZ group 2, type 2 diabetes was confirmed by measuring fasting plasma glucose and insulin levels. Blood sampled from the retro orbital plexus under diethyl ether anesthesia was used to measure plasma glucose and insulin concentration.

111 ***Heart Perfusion:***

112 Was performed according to the Langendorff technique with Krebs-
113 Heinseleit medium at a hydrostatic pressure of 55 cm H₂O and bubbled with a
114 mixture of 95% O₂ and 5% CO₂ [11].

115 ***Measurements of myocardial function***

116 Mechanical performances of the left ventricle of the heart was determined by
117 the systolic pressure, the diastolic pressure, the heart rate and the peak rate of
118 maximum left ventricular pressure rise (dp/dt) which is considered as a good index
119 of contractility. These mechanical performances were monitored during pre-
120 ischemic; ischemic and post-ischemic reperfusion phases by a balloon inserted into
121 the left ventricle and connected to a polygraph apparatus (San-ei instrument LTD
122 Nee, Tokyo, Japan). The developed pressure was calculated (systolic pressure –
123 Diastolic pressure).

124 ***Morphological Measurements:***

125 At the end of the perfusion, hearts were removed from the perfusion
126 apparatus, excess water was absorbed on tissue paper, and the heart mass was
127 weighed.

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130 **MEASUREMENT OF FASTING BLOOD INSULIN**

131 Insulin concentrations were measured in previously frozen and thawed
132 serum samples by enzyme immunoassay using the Rat Insulin ELISA kits.

133 **MEASUREMENT OF FASTING BLOOD GLUCOSE**

134 The blood glucose was assayed by the method adopted by **Trinder P (1969)**
135 [12]. The test materials for this method were supplied as kits by “Diamond
136 Diagnostics”.

137 **CALCULATION OF HOMEOSTASIS MODEL ASSESSEMENT INSULIN**

138 **RESISTANCE INDEX (HOMA-IR):**

139 Calculated as the product of fasting insulin (microunits/ml) and fasting
140 glucose (mMol/L) divided by 22.5. Lower index indicates greater insulin
141 sensitivity [13].

142 Fasting insulin (μIU/ml) × fasting glucose (mmol/L)

143 HOMA- IR = -----

144 22.5

145 **Detection of AT2R and ACE2 gene expression in rat heart:**

146 By reverse transcriptase polymerase chain reaction (RT-PCR).

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148 **Detection of MDA levels in rat heart:**

149 By the thiobarbituric acid (TBA) test.

150 **Statistical Analysis:**

151 The results were analyzed using SPSS computer software package, version
152 10.0(Chicago-IL, USA). Data were presented as mean + S.D. Differences among
153 the three groups were compared by one-way ANOVA. To study the relationship
154 between the variables, Pearson's correlation coefficient was calculated. The results
155 were considered statistically significant at $P < 0.05$.

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Results

Comparative data of the 5 studied groups

Table (1): The means $\pm$ standard deviations of the measured level of fasting blood glucose (mmol/L), fasting insulin (uIU/ml), HOMA test in the five studied groups.

Calculated parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P
Glucose (mmol/L)	4.81 $\pm$ 0.51 a	12.14 $\pm$ 1.44 b	9.45 $\pm$ 1.27 c	8.68 $\pm$ 1.79 cd	7.08 $\pm$ 0.47 d	0.00
Insulin (uIU/ml)	10.58 $\pm$ 0.57 a	18.2 $\pm$ 2.76 b	14.71 $\pm$ 1.78 c	15.13 $\pm$ 1.54 c	13.81 $\pm$ 0.46 c	0.00
Homa	2.21 $\pm$ 0.21 a	9.79 $\pm$ 2.01 b	6.10 $\pm$ 1.00 c	5.80 $\pm$ 1.28 cd	4.35 $\pm$ 0.28 d	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$.

As shown in table 1:

As expected, untreated diabetic rats (group 2) showed significant increase in the level of fasting blood glucose (mmol/L), fasting plasma insulin (uIU/ml) and HOMA-IR compared to non diabetic control rats group 1. However, these levels decreased significantly in protected groups: pioglitazone protected diabetic rats

group 3, vit E protected diabetic rats group 4 and metformin protected diabetic rats group 5 compared to untreated diabetic group 2 with no significant difference between the three treated groups. However, none of the measured parameters in the protected groups returned to control values.

Table (2): The means $\pm$ standard deviations of the measured level AT₂R and ACE₂ expression in cardiac tissue (μ g/ml homogenized tissue) in the five studied groups.

Calculated parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P
AT ₂ R mg/ml	0.18 $\pm$ 0.04 a	0.508 $\pm$ 0.06 B	0.84 $\pm$ 0.106 d	0.408 $\pm$ 0.07 b	0.503 $\pm$ 0.061 b	0.00
ACE ₂ mg/ml	0.108 $\pm$ 0.031 a	0.36 $\pm$ 0.085 B	0.72 $\pm$ 0.12 d	0.35 $\pm$ 0.026 b	0.39 $\pm$ 0.018 b	0.00
MDA nmol/mg ptn	10.93 $\pm$ 1.36 a	23.01 $\pm$ 2.68 B	16.28 $\pm$ 1.97 c	14.91 $\pm$ 1.88 c	17.45 $\pm$ 0.52 c	0.00

bearing the same initials are not statistically significant from each other at $P < 0.05$.

As shown in table 2:

The unprotected diabetic rats group 2 showed significant increase in the level of AT₂R and ACE₂ gene expression in cardiac tissue (μ g/ml homogenized

190 tissue) compared to control group 1. Pioglitazone treatment of diabetic rats almost
191 doubled the level of AT₂R and ACE₂ gene expression in cardiac tissue compared to
192 the untreated diabetic group and the other two protected groups (Vit E and
193 metformin).

194 As regards the **level of MDA in cardiac tissue (nmol/mg ptn)**: The
195 unprotected diabetic rats group 2 showed significant increase in the level of MDA
196 in cardiac tissue (nmol/mg ptn) compared to control group 1. Moreover, the
197 protected groups: PPAR γ protected diabetic rats group 3, vit E protected diabetic
198 rats group 4 and metformin protected diabetic rats group 5 showed significant
199 decrease in level of MDA in cardiac tissue compared to untreated diabetic group 2
200 although the level of the MDA remained significantly elevated compared to control
201 group 1.

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Table (3): The means $\pm$ standard deviations of left ventricular performance (developed pressure (mmHg), contractility index dp/dt(mmHg/sec) and heart rate(b/min) during the initial 30 minutes of perfusion in the five studied groups.

Calculated parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P
LVDP mmHg	114.56 ± 9.47 a	58.44 ± 5.43 b	79.44 ± 6.32 d	79.67 ± 3.39 d	59.44 ± 5.43 b	0.00
HR b/min	248.78 ± 109.11 a	224.00 ± 46.56 a	161.33 ± 18.98 b	162.44 ± 9.15 b	225.00 ± 46.56 a	0.00
dp/dt mmHg/sec	116.44 ± 7.126 a	65.78 ± 4.969 b	97.89 ± 7.688 cd	95.22 ± 8.228 d	66.78 ± 4.969 b	0.00

Groups bearing the same initials are not statistically significant from each other at $P < 0.05$.

217 As shown in table 3:

218 The unprotected diabetic rats group 2 showed significant decrease in the
219 level of LVDP (mmHg) and dp/dt (mmHg/sec) compared to control group 1 but
220 there was no significant difference in the level of HR (b/min) between the 2
221 groups.

222 Both pioglitazone protected diabetic rats (group 3) and vit E protected
223 diabetic rats (group 4) showed significant improvement in LVDP and dp/dt but
224 still not reaching control values of the nondiabetic rats. However, there was no
225 significant difference between the metformin protected diabetic rats (group 5) and
226 the unprotected diabetic rats (group 2).

227 **Table (4): The means $\pm$ standard deviations of the percentage recovery**
228 **of the left ventricle performance following ischemia-reperfusion (developed**
229 **pressure (mmHg), contractility index dp/dt(mmHg/sec) and heart rate(b/min)**
230 **in the five studied groups.**

Calculated parameter s	Group 1	Group 2	Group 3	Group 4	Group 5	P
LVDP mmHg	78.144 ± 1.93 ab	63.456 ± 4 .39 c	76.38 ± 2 . 54 b	64.28 ± 3 . 72 c	69.36 ± 0 . 71 d	0.0 0

HR b/min	77.100 ±1.90 a	65.98±7. 14 b	78.21±3. 02 a	67.63±2. 16 b	69.83±0. 83 b	0.0 0
dp/dt mmHg/sec	77.256 ±2.83 a	71.55±4. 14 b	79.56±1. 43 a	66.87±2. 90 d	69.68±0. 91 bd	0.0 0

Groups bearing the same initials are not statistically significant from each other at $P<0.05$.

As shown in table 4:

The percentage recovery of the left ventricle performance following ischemia-reperfusion (LVDP (mmHg), contractility index dp/dt (mmHg/sec) and heart rate(b/min) was significantly decreased in the unprotected diabetic rats (group 2) compared to control rats (group 1).

Regarding the protected groups of diabetic rats; PPAR γ protected diabetic rats (group 3) showed significantly higher values of percentage recovery of LVDP and **dp/dt** compared to vit E protected diabetic rats (group 4), metformin protected diabetic rats (group 5) and the untreated diabetic rats (group 2). Interestingly, pioglitazone prevented the effect of diabetes on % recovery of left ventricular performance after I/R that even returned to non diabetic control levels.

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247 ***III. Correlations between studied parameters***

248 Table (5): Correlation between the level of AT2R, ACE2 expression in cardiac
 249 tissue ($\mu\text{g/ml}$ homogenized tissue) and the level of MDA in cardiac tissue
 250 (nmol/mg ptn) with the percentage recovery of the left ventricle performance
 251 following ischemia-reperfusion (developed pressure, contractility index dp/dt and
 252 heart rate) & the correlation between the level of AT2R, ACE2 expression in
 253 cardiac tissue ($\mu\text{g/ml}$ homogenized tissue) and the level of MDA in cardiac tissue
 254 (nmol/mg ptn) :

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			AT2	ACE2	MDA
	MDA	Pearson Correlation	.088	.115	
		Sig. (2-tailed)	.491	.371	
		N	63	63	
	LVDP % recovery	Pearson Correlation	.412**	.465**	-.439**
		Sig. (2-tailed)	.001	.000	.000
		N	63	63	63
	HR % recovery	Pearson Correlation	.546**	.624**	-.363**
		Sig. (2-tailed)	.000	.000	.003
		N	63	63	63
	Dp/dt % recovery	Pearson Correlation	.610**	.603**	-.353**
		Sig. (2-tailed)	.000	.000	.003
		N	63	63	63

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268 (*) Correlation is significant at the 0.05 level (2-tailed).

269 When Pearson correlation was performed in studied groups, significant
 270 positive correlation was found between the level of AT2R expression in cardiac
 271 tissue and the percentage recovery of the left ventricle performance following
 272 ischemia-reperfusion "LVDP ($r=.412^{**}$), heart rate($r=.546^{**}$) and contractility index
 273 dp/dt ($r=.610^{**}$) & also, there was significant positive correlation between the level
 274 of ACE2 expression in cardiac tissue and the percentage recovery of the left
 275 ventricle performance following ischemia-reperfusion "LVDP ($r= .465^{**}$) and
 276 contractility index dp/dt ($r=.603^{**}$) and the percentage recovery the heart rate ($r =$
 277 $.624^{**}$). In addition, there was significant negative correlation between the level of
 278 MDA in cardiac tissue and the percentage recovery of the left ventricle
 279 performance following ischemia-reperfusion "LVDP ($r = -.439^{**}$), heart rate($r = -$
 280 $.363^{**}$) and contractility index dp/dt ($r=-.353^{**}$)".

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Discussion

282 The protective role of PPAR- γ agonist and vitamin E in diabetes was
 283 confirmed in the present study. However, the glucose level was significantly
 284 reduced in the metformin group compared to other protected groups.

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286 A recent study found that tocopherols dramatically enhanced adiponectin
287 expression and that this effect was mediated through a PPAR- γ -dependent process.
288 These findings illustrate a possible mechanistic link between vitamin E and insulin
289 sensitivity [14] It is likely that the diabetic milieu and associated changes in the
290 myocardium sensitize the diabetic heart to dysfunction after ischemic injury[15].

291 Vitamin E-protected rats showed significantly higher values of left
292 ventricular performance and contractility index.

293 Increased ROS generation may activate maladaptive signaling pathways,
294 which may lead to cell death, which could contribute to the pathogenesis of
295 diabetic cardiomyopathy [16] Increased ROS-mediated cell death could promote
296 abnormal cardiac remodeling, which ultimately may contribute to the characteristic
297 morphological and functional abnormalities that are associated with diabetic
298 cardiomyopathy[17] Increased ROS also might contribute to mitochondrial
299 uncoupling, which could impair myocardial energetics in diabetes. Strategies that
300 either reduce ROS or augment myocardial antioxidant defense mechanisms might
301 have therapeutic efficacy in improving myocardial function in diabetes mellitus
302 [18].

303 PPAR- γ agonists may exert their anti-inflammatory effects by negatively
304 regulating the expression of pro-inflammatory genes induced during macrophage

differentiation and activation. Several lines of evidence suggest that TZDs protect the heart and other organs against the tissue injury caused by ischemia/reperfusion (I/R) injury and shock [19]

The present study showed that PPAR- γ ligands improved cardiac performance after I/R. This agrees with *Shimabukuro and colleagues (1996) [20]* The authors showed that this cardioprotective effect is associated with inhibition of the JNK/AP-1 pathway [21]

Zhu and coworkers [22], showed that chronic troglitazone administration significantly improved recovery of left ventricular systolic and diastolic function as well as increasing net myocardial lactate uptake, suggesting enhanced myocardial carbohydrate oxidation [23]

A significant increase in the level of MDA in cardiac tissue was detected in the unprotected diabetic rats. However, the MDA level was equally decreased in all protected diabetic groups.

Under physiological states, most of the ROS generated within cells arises from mitochondria. Relatively few studies to date have directly measured mitochondrial ROS production in mitochondria obtained from diabetic hearts [24] ROS are produced from nonmitochondrial sources such as NADPH oxidase or

323 reduced neuronal nitric oxide synthase (NOS1) activity coupled with increased
324 activation of xanthine oxidoreductase [25] Antioxidant inhibits NF- κ B activation
325 by blocking LPS-induced I κ B degradation and nuclear translocation of the
326 cytosolic NF- κ B p65 subunit [26]

327 Potential mechanisms for cardioprotection afforded by ACE2 are unclear.
328 They include increased Ang II degradation and increased formation of Ang-(1-7)
329 which activates the G protein–coupled Mas receptor. Mas receptor activation by
330 Ang-(1-7) opposes many of the AT₁ receptor-mediated actions (vasoconstriction,
331 hypertrophy, fibrosis), thereby improving cardiac function and remodeling and
332 attenuating heart failure. Mas receptors are also localized to cardiac myocytes and
333 activate NO production [27].

334 In the present work, % recovery of cardiac performance after I/R injury was
335 positively correlated with levels of AT2R and ACE2 gene expression and
336 negatively correlated with levels of MDA.

337 It has been shown that TZDs could inhibit the oxidative stress indirectly
338 through the inhibition of the promoter activity of the chemokine genes and the
339 inhibition of NF- κ B activation on these promoter with the inhibition of the
340 expression of proinflammatory adhesion molecules (ICAM-1, VCAM-1, E-
341 selectin)[32]. Moreover PPAR- γ agonists prevent the phosphorylation of NF- κ B

342 p65 and inhibit the activities of NF- κ B and AP-1 and thus reducing the generation
343 of oxygen-derived free radicals in diabetic hearts [28].

344 In our study, lowering glucose level by metformin induced significant
345 decrease in MDA. Metformin prevent ROS formation by preventing the build-up
346 of mNADH through insulin-stimulated nutrient uptake and preventing the
347 entrance of energetic substrates (pyruvate, fatty acids) into the mitochondria [29]
348 Furthermore, metformin has been shown to reduce ROS levels in human
349 leukocytes by either directly scavenging the free radicals or modulating their
350 intracellular production [30].

351 Comparing the levels of HOMA-IR and cardiac tissue MDA in our studied
352 groups confirms the role of oxidative stress in pathophysiology of diabetic hearts
353 and also confirms the antioxidant effect of PPAR- γ agonists which surprisingly
354 enough was not different from either vitamin E or metformin.

355 The unprotected diabetic rats showed significant increase in the level of
356 AT₂R and ACE₂ gene expression. This might indicate a protective role for both
357 AT₂R and ACE₂ [31]

358 The levels of AT₂R and ACE₂ gene expression in cardiac tissue were
359 significantly elevated in pioglitazone protected groups compared to other groups.

360 There is good evidence that PPAR- γ agonists mediate inhibition of the AT1R
361 expression as well as Ang II-mediated signaling pathways which may explain the
362 increase in the expression of AT2R and ACE2 [32,33]

363 *Molavi et al., (2006)* [34] reported a reduction of infarct size, attenuation of
364 myocardial AT1R and increase in AT2R mRNA expression in the TZDs group.

365 Taken together, these results clearly suggest a cross talk between ROS, PPAR
366 - γ and RAS systems which interplay in the pathogenesis of diabetes and its cardiac
367 complications.

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