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GENE EXPRESSION OF ENDOTHELIN-1 IN THE RAT PLACENTA UNDER CHRONIC FETOPLACENTAL HYPOXIA INDUCED BY UTERINE ARTERY LIGATION

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A vasoactive peptide, endothelin-1 (ET-1) has been identified in the mammalian placenta. Its increase in the fetal circulation was demonstrated not only in acute but also in chronic fetal hypoxia in human. The aim of this study was to examine the effect of chronic fetoplacental hypoxia induced by uterine artery ligation on ET-1 gene expression in the rat placenta. Unilateral uterine artery ligation was performed to the pregnant Sprague-Dawley rats on Day 18 of gestation and the pregnancy was terminated on Day 21 of gestation. The effect of maternal starvation on the placental ET-1 messenger ribonucleic acid (mRNA) levels was also examined for comparison with the same time period. Relative abundance of the placental ET-1 mRNA was determined by quantitative reversed transcriptase polymerase chain reaction coupled with Southern blotting. Both maternal starvation and uterine artery ligation significantly reduced fetal and placental weight. In contrast, the placental ET-1 mRNA levels increased 2-fold by the uterine artery ligation whereas those in the maternal starvation group did not. Thus, it is unlikely that the reduced meterno-fetal transfer of nutrients by the uterine artery ligation could enhance the placental ET-1 gene expression. These results suggest that the enhanced placental ET-1 gene expression upon chronic fetoplacental hypoxia may contribute to the pathophysiology of the placental circulation in the fetal growth retardation.

Introduction

It is well known that placental circulation is regulated by humoral factors because it lacks autonomic innervation[1)2)]. Among these humoral factors, endothelin-1 (ET-1), a potent vasoconstrictive peptide produced by endothelial cells, has been shown to have profound vasoconstrictive effect on placental vessels in vitro[3)4)]. Presense of ET-1 was shown in trophoblastic cells of the human placenta[5)], in endothelial cells of capillaries, arteries, and veins in the labyrinth zone of the rat placenta[5)]. Increased vascular tone in the placental vascular tree has been demonstrated in human fetal growth retardation with maternal pregnancy-induced hypertension. In pregnancy-induced hypertension, reduced oxygen supply to the fetus through the placenta is of pathophysiological importance in the fetal growth retardation. It is intriguing if ET-1 produced in response to tissue hypoxia in the placenta could regulate the placental vascular tone. Ferri et al. reported that circulating ET-1 levels were elevated in patients with chronic hypoxia due to chronic obstructive pulmonary disease[6)]. Along

with the circulating levels of ET-1 in relation to hypoxia, enhanced ET-1 production was also shown at tissue level. Dikranian et al. demonstrated enhanced immunolabelling of ET-1 in both endothelial and epithelial cells of the large intestinal mucosa of the rats under chronic hypoxia[7].

We have previously demonstrated that ET-1 levels were elevated in human fetal circulation upon acute fetal hypoxia and this increase was associated with hemoconcentration of the neonate suggesting blood shift from the placenta to the fetus occurred by the action of ET-1[8]. In addition, it was reported that in adult rat lung, hypoxic exposure was associated with increases in ET-1 messenger ribonucleic acid (mRNA) and ET_A receptor mRNA[9]. However, little is known about the roles of ET-1 in chronic fetoplacental hypoxia in which fetal growth retardation occurs along with altered placental circulation. It has been shown that mammalian placentas of human, sheep and rat express ET-1 gene[9]~[12] and its receptor genes[13].

We have examined gene expression of ET-1 in rat placenta in response to chronic fetoplacental hypoxia. Here we report that chronic fetoplacental hypoxia upregulates ET-1 gene expression in the rat placenta and this augumented expression of ET-1 gene is not observed in fetal growth retardation produced by maternal starvation.

Materials and Methods

1. Animals and tissue preparation

1) Uterine artery ligation experiment

Timed Sprague-Dawley (SD) rats (Charles River Breeding Laboratories, Tokyo, Japan) were housed in individual cages in a temperature (22°C) and humidity-controlled room with a 12-hour photoperiod (8 AM to 8 PM). Animals had free access to tap water and were fed rat chow pellets. On Day 18 of gestation, rats were anesthetized with intraperitoneal administration of 0.01 mg/kg of pentobarbital and the abdomen was opened along the midline. The suture was placed around either side of the uterine artery and tied before closing the abdomen and the animals allowed to recover[14]. After 6, 12, 24, 48 and 72 h from the ligation, the animals were again anaesthetized with pentobarbital and placentas and fetuses were removed. The rats were then immediately killed by injection of 50 mg pentobarbital i.p. Rats were only included in the experiment if they had between 5 and 8 fetuses in each horn. The placentas were immediately stored at -80°C until RNA extraction. The fetuses and the placentas from the unligated horn were served as controls.

2) Maternal starvation experiment

The same timed pregnant SD rats were fed only on water from Day 18 until Day 21 of gestation. Control rats were fed ad libitum as described above. On Day 21, the fetuses and the placentas were obtained with the same protocol as in the uterine artery ligation experiment.

All animal experiments were conducted in accordance with the American Physiological Society guidelines and were approved by the Institutional Animal Care and Use Committee of the Tokyo Women's Medical College, Tokyo, Japan.

2. RNA isolation and Southern blot analyses

Tissue RNA was extracted from the organs by the acid guanidine isothiocyanate-phenol-chloroform method[15]. The total RNA (2.5 μg) was used for cDNA synthesis. First-strand cDNA synthesis was primed with $oligo(dT)_{12-18}$ and carried out according to manufacturer's specifications (SuperScript Preamplification System for First Strand cDNA Synthesis, Gibco BRL). GAPDH gene served as a constitutively expressed internal control for cDNA quantity and integrity. The primer oligonucleotide sequences were designed as described previously[16][17]. The sequence of forward primer, 5′GTT, GCT, CCT, GCT, CCT, CCT, TG3′, corresponding to nucleotides 339-358 of the published synthesized ET-1 cDNA sequence, and a reverse primer, 5′GCA, TGG, AGA, GCG, CAG, AGT, TG3′, corresponding to nucleotides 851-870 of the ET-1 sequence were synthesized

respectively. The 5′ and 3′ primers and Taq DNA polymerase (2.5 mM) were added to the PCR mixture. The resultant cDNAs were amplified for 40 cycles using a Perkin Elmer Cetus DNA Thermal Cycler employing a step program (90°C, 50 sec [melt]; 60°C, 105 sec [anneal]; 72°C, 120 sec [extend]; 72°C, 10 min [final extension]). In preliminary experiments, the influence of the number of amplification cycles on the yields of PCR products was assessed. The PCR reaction products became visible on ethidium bromide staining from 20 cycles in ET-1 and from 30 cycles in GAPDH. Both PCR products showed linear increase up to 50 cycles by ethidium bromide staining.

The PCR products were separated in 1% agarose gels and blotted overnight onto a nylon membrane with 20× SCC. ET-1 cDNA probe: A 0.5-kb rat preproET-1 cDNA probe was kindly provided by Dr. Tom Quertermous (Vanderbilt University, Nashville, TN). This probe contains most of the coding sequence of preproET-1 cDNA and does not cross-hybridize with ET-2 or ET-3 mRNA.

The cDNA probes were radiolabeled to a specific activity of 10^8～10^9 counts/min /μg with the random hexamer primer labeling method. The radiolabeled ET-1 probe was separated from unincorporated nucleotides using Quick Spin columns containing G-50 Sephadex (Boehringer Mannheim, Indianapolis, IN). After hybridization, blots were washed in 500 ml 2× SSPE/0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min and twice in 1× SSPE/0.1× SDS at 60°C for 20 min. Blots were slightly dried and autoradiographed to X-ray film (Kodak X-OMAT film, Sigma), densitometrically corrected by that of the GAPDH.

3. Statistical analysis

Results were expressed as means±SE. Statistical comparisons of the placental ET-1 mRNA levels were performed using Student's unpaired t test. Differences were reported as significant if $p<0.01$.

Results

Following unilateral uterine artery ligation, fetal weight was 28% lower ($p<0.01$) than that of fetuses from the sham operated horn, fetal length was 18% shorter ($p<0.05$) and placental weight was 25% lower ($p<0.01$) on Day 21 of gestation (Table 1). As with the uterine artery ligation, maternal starvation resulted in fetal growth retardation; 21% deficit in fetal weight ($p<0.01$), 16% deficit in fetal length ($p<0.01$) and a 17% deficit in placental weight ($p<0.01$) on Day 21 of gestation (Table 2). Figure 1 and 2 shows a time course study on the effect of uterine artery ligation on fetal weight and placental weight, respectively. In sham operation group, the fetal growth showed acceleration between Day 19 to Day 20 and thereafter it slowed down during the next 24 h. In contrast, this acceleration in the fetal growth spurt was abolished by the ligation of uterine artery. Placental growth showed different pattern from that of the fetus. In sham operation group,

Table 1 Effect of unilateral uterine artery ligation of fetal and placental growth

	Sham operation (n=31)	Uterine artery ligation (n=31)
Fetal body weight (g)	4.45±0.001	3.35±0.001*
Fetal length (cm)	3.92±0.001	3.20±0.001*
Placental weight (g)	0.49±0.010	0.41±0.001*

On Day 18 of gestation, unilateral uterine artery ligation was placed. On Day 21, fetuses and placentas were removed for analyses. Data were shown in mean±SE. Asterisk disignates $p<0.01$.

Table 2 Effect of maternal undernutrition of fetal and placental growth

	Control (n=20)	Undernutrition (n=20)
Fetal body weight (g)	5.62±0.07	4.43±0.17*
Fetal length (cm)	4.21±0.01	3.79±0.07*
Placental weight (g)	0.54±0.03	0.45±0.03*

Pregnant rats were fed on either usual foods or water only from Day 18 to Day 21 of gestation. Data were shown in mean±SE. Asterisk designates $p<0.01$.

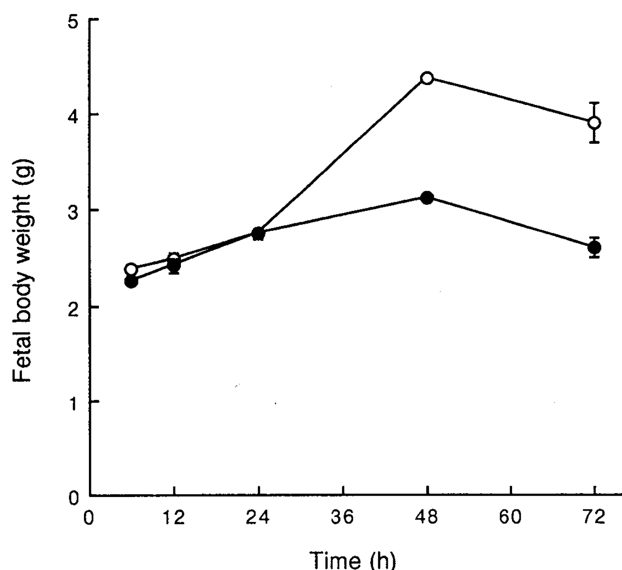

Fig. 1 Time course study on fetal body weight after unilateral uterine artery ligation
After placing unilateral uterine artery ligation, fetuses were removed at 6, 12, 24 (Day 19), 48 (Day 20) and 72 h (Day 21) after the ligation. Closed circle designates the uterine artery ligation group. Data were shown in mean±SE. N=20/each time point.

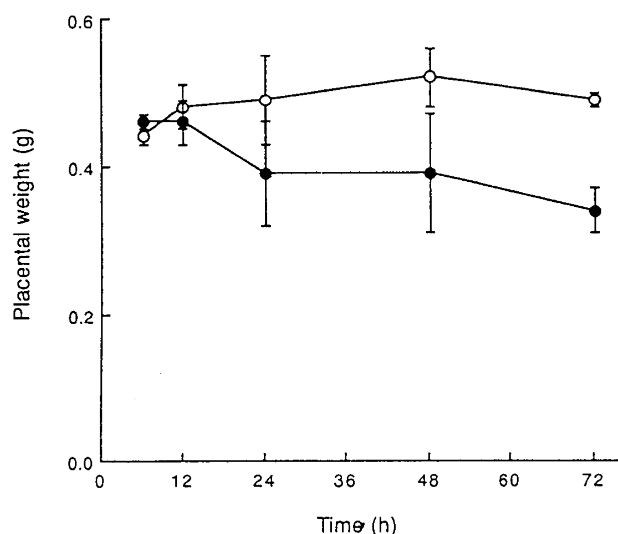

Fig. 2 Time course study on placental weight after unilateral uterine artery ligation
After placing unilateral uterine artery ligation on Day 18 of gestation, placentas were removed at 6, 12, 24 (Day 19), 48 (Day 20) and 72 h (Day 21) after the ligation. Open circle designates the sham operation group. Closed circle designates the uterine artery ligation group. Data were shown in mean±SE. N=20/each time point.

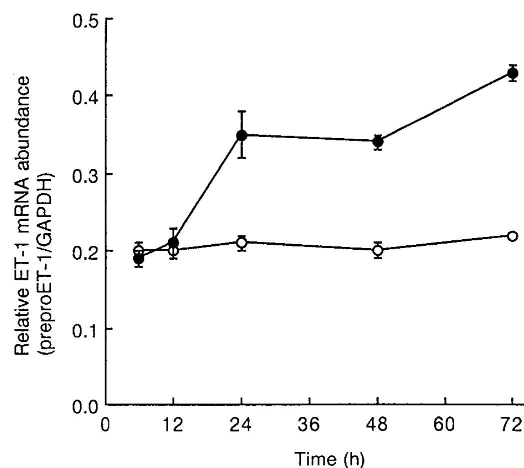

Fig. 3 Time course study on ET-1 mRNA relative abundance in the placenta after unilateral uterine artery ligation
After placing unilateral uterine artery ligation on Day 18 of gestation, placentas were removed at 6, 12, 24 (Day 19), 48 (Day 20) and 72 h (Day 21) after the ligation. Closed circle designates the uterine artery ligation group. Data were shown in mean±SE. N=4 to 5/each time point.

the placental weight increased gradually and reached plateau on 12 h after the operation whereas it decreased at 24 h after the ligation of uterine artery. Thereafter, it showed steady decrease up to the delivery of the fetuses on Day 21. In accordance with the slow increase of the placental weight, ET-1 mRNA relative abundance showed slight increase in the sham operation group whereas it showed remarkable increase at 24 h after the ligation of uterine artery and this increase was sustained until delivery (Figure 3). To determine whether hypoxia or nutritional deprivation could affect gene expression of ET-1 in the placenta, we examined ET-1 mRNA levels in the placentas of the maternal starvation for comparison. Although growth retardation was observed in both groups, placental ET-1 mRNA levels did not show any difference in the placentas subjected to the maternal starvation whereas it showed two-fold increase ($p<0.01$) in those of uterine artery ligation (Figure 4, 5).

Discussion

In the present study, we have demonstrated

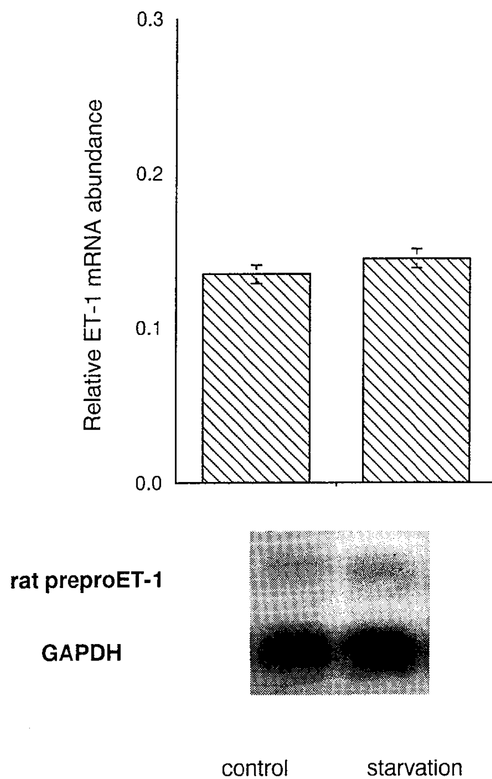

Fig. 4 Effect of maternal starvation on relative ET-1 mRNA abundance in the placenta
Relative ET-1 mRNA abundance of the placentas (n=4) from the maternal starvation experiments is shown. Pregnant SD rats were fed only on water from Day 18 until Day 21 of gestation. Control rats were fed ad libitum. On Day 21, the fetuses and the placentas were obtained with the same protocol as in the uterine artery ligation experiment. Data were shown in mean±SE. Representative autoradiogram is shown in the lower column.

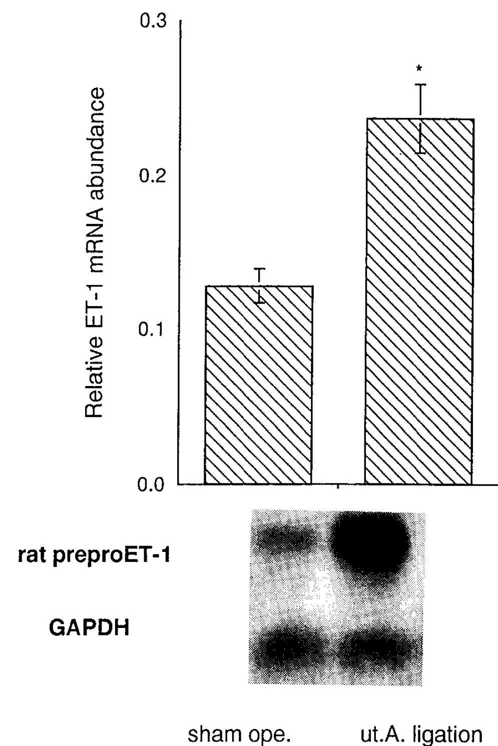

Fig. 5 Effect of uterine artery ligation on relative ET-1 mRNA abundance in the placenta
Relative ET-1 mRNA abundance of the placentas (N=25) from the uterine artery ligation experiments is shown. Data were shown in mean±SE. Asterisk designates $p<0.01$. Representative autoradiogram is shown in the lower column.

that the increase of ET-1 gene expression in rat placenta upon chronic fetoplacental hypoxia by unilateral uterine artery ligation. Since we wanted to simulate human intrauterine growth retardation (IUGR) observed in placental insufficiency, we made utero-placental circulatory disturbance by restricting uterine bloody supply on one side of the bicornuate uterus of the pregnant rats. It has been known that IUGR related to preeclampsia shows asymmetric growth in which brain growth is minimally disturbed in compensation for variable degree of growth retardation of other nonvital organs. By placing uterine artery ligation at Day 18 of gestation in the pregnant rats, we could mimic the asymmetric growth retardation seen in human IUGR (data are not shown) as Philip W et al. reported previously[18]. It is noteworthy that the uterine artery ligation interfered increase in the placental weight at frist place as early as 24 h after the ligation when the fetal weight showed no difference. Our observation that the reduction in the placental weight proceeded the fetal growth stunt by 48 h may suggest that the placental dysfunction is a rate-limiting factor in the pathophysiology of IUGR. Although whether this decrease in the placental weight means disturbance in placental growth or simply decrease in blood content in the placenta is not shown from our study, the fact that the changes in the placental ET-1 mRNA levels coincided with that of the placental weight suggests ET-1 gene expression in the placenta might be closely related to the changes in placental circulation. Since it is generally

accepted that ET-1 has a potent long-acting vasoconstricting effect in the human placental villous vasculature tree in vitro[19], increase of ET-1 production in response to hypoxia might induce constriction of the vascular tree in the placenta.

The other important cause which affects fetal growth is maternal malnutrition. It is generally accepted that symmetric growth retardation occurs if nutritional supply is limited due to maternal malnutrition[20]. To rule out the possibilities that the enhanced expression of ET-1 gene in the placenta is rather a non-specific phenomenon when the fetal growth is restricted, or alternatively, reduction in the placental transfer of nutrients may trigger the ET-1 gene expression, we examined the placental ET-1 mRNA levels under maternal starvation of rats. In contrast to those of the uterine aretery ligation, placental ET-1 mRNA levels did not change in the growth retardation of the maternal starvation. These results support the idea that hypoxic stimulus plays key role in ET-1 gene expression. From a time course study on ET-1 gene expression in cultured human umbilical vein endothelial cells, hypoxia caused increase in ET-1 mRNA levels dramatically from 24 to 48 h after exposure to hypoxia[21]. This time period is in accordance with our data demonstrating that placental ET-1 mRNA started to increase from 48 h after uterine artery ligation.

In addition, the other factor(s) that could be induced by hypoxia may also induce ET-1 gene expression. Under hypoxia, decrease in blood flow in the placenta could allow thrombin formation[22]. Thrombin, in turn, could further stimulate ET-1 gene expression as reported in vitro study on human umbilical vein endothelial cells in culture[23].

The role of ET-1 in the placenta under chronic fetoplacental hypoxia is not known. Besides vasoconstrictive effect, ET-1 has a growth promoting effect on human first trimester placental fibroblast[24]. Thus, locally produced ET-1 under hypoxia may modulate the growth of mesenchymal cells in the placenta. Recently, Kurihara et al. demonstrated that overexpression of the ET-1 gene in trangenic mice resulted in birth of growth retarded mice (personal communication from Dr. Kurihara, University of Tokyo), suggesting ET-1 has a suppressive effect on fetal growth via vasoconstriction of the placenta and/or the fetal circulation.

From these observations, it is suggested that major role of ET-1 in chronic fetoplacental hypoxia is to constrict placental vessels. Relationship between ET-1 gene expression and hypoxia has been demonstrated in the hypoxic pulmonary vasoconstrictor response (HPVR) where increased ET-1 mRNA levels in the hypoxic lung tissue were negatively correlated to the pulmonary blood flow[9]. As in the HPVR, hypoxia-induced fetol-placental vasoconstriction may shunt blood away from hypoxic regions to the regions with better oxygenation thus improving net gas exchange in the placenta[25].

We suggest that placental ET-1 gene expression plays crucial role(s) in the local paracrine/autocrine control of placental circulation upon chronic fetoplacental hypoxia.

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妊娠ラット子宮動脈結紮による慢性胎児胎盤低酸素症における胎盤のエンドセリン遺伝子発現の検討

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エンドセリン-1（ET）は培養血管内皮細胞より発見された強力な血管収縮性ペプチドであり，低酸素環境下でその産生が増加することが知られている．ET はその受容体とともに胎盤で発現することから，慢性胎児低酸素症の胎盤血流調節因子としての ET の関与を明らかとするために，ラット胎盤での ET 遺伝子発現を検討した．

今回我々は，妊娠18日齢の Sprague-Dawley ラットの片側の子宮動脈を結紮し，非結紮側を対照として結紮の6，12，24，48，72時間後に胎盤を摘出後，RNA を抽出した．RT-PCR 法によるサザンブロット解析により ET 遺伝子発現を検討した，また，比較として母体低栄養による胎児発育遅延を妊娠18日から21日までの72時間，水分のみを与えることによる飢餓により作製し，胎盤の ET 遺伝子発現を同様に検討した．

その結果，子宮動脈結紮後，胎仔体重，ならびに胎盤重量は24時間以降で減少を示し，72時間ではそれぞれ対照の62%（$n=31$，$p<0.01$），75%（$n=31$，$p<0.01$）となった．一方，母体低栄養では胎仔体重，ならびに胎盤重量はそれぞれ対照の79%（$n=20$，$p<0.01$），83%（$n=20$，$p<0.05$）と減少した．ラット胎盤の ETmRNA relative abundance（preproET-1/GAPDH; mean±SEM）は慢性胎児低酸素症モデルでは対照群と結紮群でそれぞれ0.128±0.011 vs 0.237±0.022（$p<0.01$）と結紮群で約2倍の有意の増加を示した．一方，母体低栄養モデルでは胎盤の ET mRNA relative abundance は対照群と低栄養群とでそれぞれ0.135±0.010，0.145±0.006と差を認めなかった．

以上より，子宮動脈結紮によって惹起された慢性胎児低酸素症モデルにおいて，胎盤の ET 遺伝子発現の増加を確認した．子宮動脈結紮により母体からの胎児への栄養の物質輸送の障害が胎盤の ET 遺伝子発現に関与している可能性は，母体低栄養による IUGR において胎盤の ET 遺伝子発現に差が認められなかったことから否定的と考えられた．以上より，ET は低酸素負荷に反応して胎盤局所で産生，放出される autocrine あるいは paracrine 因子として胎盤血管の収縮にあずかっていると考えられた．