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Review

G2A as a receptor for oxidized free fatty acids

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ABSTRACT

G2A was identified as a G protein-coupled receptor that can be induced by different classes of DNA-damaging agents and block cell cycle progression in lymphocytes. We recently reported that G2A functions as a receptor for oxidized free fatty acids derived from linoleic and arachidonic acids. When ectopically expressed in CHO cells, G2A mediates intracellular signaling events such as intracellular calcium mobilization and JNK activation in response to oxidized free fatty acids. In human epidermal keratinocytes, G2A mediates the secretion of cytokines including interleukin-6 and -8, and blocks cell cycle progression at the G1 phase in response to ligands. G2A might function as a sensor that monitors the oxidative states and mediates appropriate cellular responses such as secretion of paracrine signals and attenuation of proliferation.

Contents

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1. Introduction

G2A was identified as one of the genes that are induced in pre-B lymphocytes by overexpression of BCR-ABL, a chimeric tyrosine kinase oncogene generated by a reciprocal chromosomal translocation [1]. There are two striking features in G2A: it can be induced in lymphocytes by different classes of DNA-damaging agents, and it can block cell cycle progression at the G2/M phase.

Controversial findings have been reported regarding the endogenous ligands of G2A. Lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC) were once reported to be potent ligands for G2A [2], but crucial binding data could not be reproduced, leading to the retraction of the paper [3]. However, many papers have reported G2A-dependent LPC effects. Another report showed that G2A has proton-sensing activity to enhance inositol phosphate (IP) turnover in response to acidic pH, and that this activity is antagonized by LPC [4]. However, G2A is less sensitive to pH fluctuations than the other three proton-sensing G protein-coupled receptors (GPCR) forming a homologous cluster, that is, OGR1, GPR4 and TDAG8 [5–8].

We identified that G2A also functions as a receptor for oxidized free fatty acids such as 9-hydroxyoctadecadienoic acid (HODE) and 11-hydroxyeicosatetraenoic acid (HETE) [9], which has also been observed by another group [10]. We further reported the pro-inflammatory roles of G2A in human keratinocytes during oxidative stress [11]. In this review article, we summarize the characteristics of G2A as a receptor for oxidized free fatty acids including our recent unpublished data, and discuss the putative physiological functions of G2A.

2. Identification of G2A as a receptor for oxidized free fatty acids

In 2000, we launched a project to identify a novel lipid mediator by screening ligands of orphan GPCRs. At that time, there were more than 100 non-odorous GPCRs whose ligands had not been identified. We selected 10 orphan GPCRs that were likely to have lipid ligands based on phylogenetic tree analysis. G2A showed similarity to OGR1, TDAG8 and GPR4. OGR1 was reported to be a receptor for SPC in 2000 [12], then G2A for SPC and LPC in 2001 [2]. However, we kept these receptors for the targets of ligand screening, because we could not reproduce these reports in our assay system. After that, both of the reports were retracted due to problems in reproducing the ligand binding data [3]. However, many reports have shown that LPC has regulatory or modulatory effects on G2A.

For the ligand screening of the 10 selected orphan receptors, we established clones that stably overexpressed each of the receptors in CHO cells. The cell surface expression of each receptor was confirmed by staining FLAG-epitope tag added to the N-terminus after the first methionine. Ligand screening assay was performed based on intracellular calcium mobilization using a lipid library that consists of 203 lipids as ligand sources. As a result, we observed that some of the lipids specifically evoked calcium responses in G2A-expressing cells.

2.1. Ligand specificity of G2A

Of the lipids that evoked calcium responses in G2A-expressing cells, 9-HODE had the highest activity (Fig. 1). 9-HODE is an oxidized fatty acid derived from linoleic acid. The calcium increases elicited by 9-HODE were observed at the concentration above 100 nM, and reached a plateau at around 10 μM. The concentration of 9-HODE required to induce the half-maximum response was about 0.5 μM. 9-Hydroperoxyoctadecadienoic acid (HPODE) showed slightly weaker activity, and their stereoisomers 13-HODE and 13-HPODE showed much weaker activity.

Of the arachidonic acid-derived oxidized fatty acids, 11-HETE was the most potent ligand, and was comparable with 9-HODE. Other HETEs (5-, 8-, 9-, 12- and 15-HETE) also evoked calcium increases with activity at around half of 9-HODE or 11-HETE. As shown in Fig. 1, the structure from the ω-end to the hydroxy-moiety of 9-HODE and 11-HETE is identical, suggesting that G2A recognizes the length of the carbon chain and the position of the hydroxy-moiety. When 9-HODE was esterified with cholesterol, the activity was not detected. Neither LPC nor SPC showed any activity at concentrations of up to 10 μM in our assay conditions.

Chiral selectivity of G2A is not strict. When 9(*S*)- and 9(*R*)-HODE were compared, 9(*S*)-HODE showed only slightly higher activity than 9(*R*)-HODE. Various kinds of racemic HETEs also activated G2A. These results suggest that G2A works as a receptor for oxidized free fatty acids, not only for enzymatically oxidized ones, but also for non-enzymatically oxidized ones during various oxidative stress.

2.2. Differences between human G2A and mouse G2A

In contrast to human G2A, mouse G2A did not respond to either oxidized free fatty acids, or to LPC in our assay conditions (unpublished observation). We attempted to identify mouse G2A ligands by the same strategy as for human G2A, but we have not succeeded so far. Differences between human and mouse G2A were also reported for the proton-sensing activity [8]. While human G2A responded to acidic pH, mouse G2A did not.

Human and mouse G2A have about 67% overall identity in the amino acid sequence. Although they have similar transmembrane domains (79% identity), they are rather divergent in the N-terminal extracellular domain (21%), intracellular loops (67%), extracellular loops (50%), and C-terminal cytoplasmic tail (41%). These relatively high divergences may explain the differences in ligand specificity or intracellular signaling efficiency between human and mouse G2A.

G2A knockout mice were reported to develop a late-onset autoimmune syndrome [13]. Mouse G2A was also reported to be involved in atherosclerosis [14,15]. The identification of ligand(s) for mouse G2A would improve our understanding of the roles of G2A in these pathological conditions.

2.3. Intracellular signaling pathways evoked by 9-HODE

In CHO cells that overexpress G2A, 9-HODE evoked increases in intracellular calcium in a dose-dependent manner. Pertussis toxin pre-treatment inhibited the response by half. This indicates that G2A mediates the intracellular calcium increase by coupling both with G_i and with pertussis toxin-insensitive G_q protein. 9-HODE did not evoke cAMP accumulation. Instead, 9-HODE inhibited forskolin-induced cAMP production in a pertussis toxin-sensitive manner. In the GTPγS binding assay using membrane fractions from HEK293 cells, 9-HODE-induced GTPγS binding was clearly observed by co-expression of G2A with G_i. 9-HODE-induced GTPγS binding was also observed by co-expression with G_{13}, but not with G_{16}. These results indicate that G2A can couple with G_i, G_q and G_{13} types of Gα protein in CHO or HEK293 cells. The type of Gα protein that couples with G2A may vary depending on the cellular context [16].

In many cases, activation of GPCR by ligand stimulation leads to activation of the MAP kinase family. Of three major MAP kinases examined (i.e., ERK1/2, p38 and JNK), 9-HODE induced modest activation of JNK that peaked at around 10 min in CHO cells that overexpressed G2A. On the other hand, activation of ERK1/2 and p38 was not observed. It remains to be elucidated how G2A activates JNK and what the biological consequence of JNK activation is.

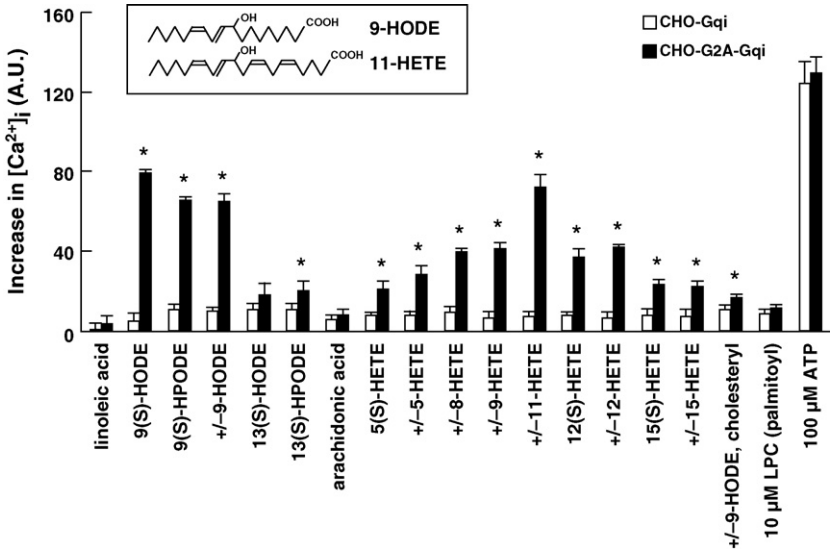

Fig. 1. Ligand specificity of G2A. CHO-G_{qi} (open square) and CHO-G2A-G_{qi} (filled square) cells were stimulated with various oxidized derivatives of linoleic and arachidonic acids, 1 μM each, and increases in intracellular calcium concentration were measured (mean + S.D., $n = 4$). G_{qi} is a chimeric protein in which nine C-terminal peptides of G_q protein were replaced with corresponding residues of G_i protein, and was utilized for enhancing calcium responses. Structures of 9-HODE and 11-HETE are shown in the inset. Asterisk, $p < 0.01$ (Student's t-test) compared with the corresponding values of the CHO-G_{qi} cells [9].

2.4. Proton-sensing activity of G2A

LPC was reported to have an antagonistic effect when G2A was activated by protons [4]. We examined the effects of LPC on 9-HODE-induced G2A activation, but pre-treatment and co-stimulation with LPC did not alter the intensity of 9-HODE-induced intracellular calcium increases in CHO cells.

When exposed to acidic pH, G2A-expressing CHO cells showed a much smaller increase in accumulation of IP than when the cells were stimulated with 9-HODE. When co-stimulated with 9-HODE and acidic pH, G2A mediated just an additive increase in IP accumulation. This suggests that 9-HODE and proton involve different mechanisms to activate G2A and do not interfere with each other.

Some histidine residues in the N-terminal extracellular region and extracellular loops were found to be critical for proton-sensing activity in OGR1, GPR4 and TDAG8 [5,6]. However, G2A has other basic amino acids such as lysine and arginine in the corresponding positions in the N-terminal region. The dissociation constant with a proton in the side chain of histidine is pH 6.04, which enables histidine to sense pH changes in the physiological range. In contrast, those of lysine and arginine are much higher, and almost all these residues are protonated at physiological pH. Thus it has been attributed to these amino acid differences that G2A has a lower proton-sensing activity and constitutively higher IP accumulation at physiological pH than the other proton-sensing GPCRs [17].

2.5. Production of oxidized free fatty acids in vivo

2.5.1. Enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids

Polyunsaturated fatty acids serve not only as structural components but also as precursors for various bioactive lipid mediators and as receivers for reactive oxygen species. For example, arachidonic acid is a precursor for prostaglandins, leukotrienes and HETEs. Esterified linoleic acid species are one of the major components of low-density lipoprotein (LDL) and are continuously exposed to many kinds of oxidative stress. It was proposed that circulating 9/13-H(P)ODE levels can be used as markers for evaluating oxidative stress [18]. Membrane phospholipids are also the targets of reactive oxygen species derived from various intracellular processes such as the respiratory burst and mitochondrial oxidative phosphorylation [19]. When polyunsaturated fatty acids are attacked by reactive oxygen species, lipid peroxy radicals are produced, which triggers further radical chain reactions leading to the production of various lipid hydroperoxides. As a result of subsequent reduction by glutathione peroxidase, 9- and 13-HODE are produced from linoleic acid, and 5-, 8-, 9-, 11-, 12- and 15-HETE from arachidonic acid with varying stereoconfiguration (*S* or *R*).

Prostaglandin endoperoxide H synthase (cyclooxygenase) and lipoxygenase are well known enzymes that oxygenate arachidonic acid. As for HETE production, 5-, 12- and 15-lipoxygenase have already been identified in human [20], but lipoxygenase which specifically produces 11-HETE has not been reported. 11-HETE can be produced as a by-product of cyclooxygenase activity converting arachidonic acid to prostaglandin G_2 [21,22].

While 13-HODE can be produced from linoleic acid by the action of 12/15-lipoxygenases [23,24], 9-HODE production by lipoxygenases has not been reported in human. Mouse 8-lipoxygenase was identified and found to convert linoleic acid to 9-HODE [25], but its human homolog is 15-lipoxygenase 2 which cannot produce 9-HODE [25,26]. Although it is also known that cytochrome P450 enzymes are involved in the metabolism of linoleic and arachidonic acids to generate various hydroxy and epoxy fatty acids including HODEs and HETEs [27,28], 9-HODE and 11-HETE appears to be produced mainly by non-enzymatic oxidation.

2.5.2. Liberation of oxidized free fatty acids

Most polyunsaturated fatty acids exist in vivo in esterified forms such as phospholipid and cholesterol ester. However, G2A does not recognize esterified 9-HODE. Thus oxidized fatty acids should be liberated before they can function as G2A ligands. As polyunsaturated fatty acids are usually esterified with glycerophospholipid at the *sn*-2 position, phospholipase A_2 may be responsible for the production of G2A ligands. Calcium-independent PLA_2s are involved in the remodeling of membrane phospholipids, and can hydrolyze oxidized phospholipids, although it is controversial, owing to methodological difficulties, whether calcium-independent PLA_2s prefer oxidized phospholipids to native ones as substrates [29]. Among nine different secretory PLA_2s in human, group V and X secretory PLA_2s were found to induce potent hydrolysis of

phosphatidylcholine in LDL or in plasma membrane [30,31], leading to the production of large amounts of free fatty acids and LPC. It is possible that these secretory PLA_2s are released in response to oxidative stress from immune cells such as macrophages and mast cells, and hydrolyze oxidized phospholipids. Similarly, the cholesterol ester of oxidized fatty acids could be hydrolyzed by lysosomal esterases secreted from macrophages.

The other candidate responsible for the liberation of oxidized free fatty acids is plasma-type platelet activating factor acetylhydrolase (also termed as group VII PLA_2 [32]). This enzyme is mainly associated with LDL and high-density lipoprotein in plasma, and is believed to hydrolyze acetyl or truncated short-chain fatty acids at the *sn*-2 position in phospholipids [33]. Recently, it was reported that oxidized phospholipids are also good substrates for this enzyme [34]. Further studies are needed to clarify the precise mechanism of production of G2A ligands.

3. Identification of an alternative splicing variant of G2A

One of the striking features of G2A is that it is a stress-inducible receptor. To better understand the physiological roles of G2A, it will be useful to elucidate how G2A is induced by various kinds of stress. During our research on the transcriptional regulation of G2A, we identified an alternative splicing variant. Here we briefly describe our recent unpublished data on the splicing variant. The complete sequence of mRNA has been deposited on DNA Data Bank of Japan (accession number: AB465600), and we are now preparing the manuscript for publication.

3.1. Structure of the G2A gene

According to the data in NCBI, the G2A gene (Gene ID: 29933) is located on chromosome 14q32.3, and consists of four exons. The coding region starts near the 3′-end of the exon 3, and ends in exon 4. During 5′-RACE analysis to determine the transcription initiation site, we found that there is an alternative splicing variant that lacks exon 3. In this variant, there is an in-frame start codon at the 3′-end of exon 2. As a result of 5′- and 3′-RACE analysis, these two alternative splicing variants were found to utilize the same transcription initiation site and polyA signal. We designated the newly identified splicing variant G2A-b, and the original one G2A-a. G2A-a and G2A-b encode 380 and 371 amino acids, respectively. The N-terminal eleven residues from exon 3 in G2A-a are replaced with two residues from exon 2 in G2A-b. We also confirmed the expression of these two variants by northern blot analysis in HL-60 cells.

3.2. Tissue distribution and induction of G2A-a and G2A-b

There was no obvious difference in the tissue distribution between G2A-a and G2A-b when examined by real-time PCR analysis using a human cDNA panel as the template. Peripheral blood leukocytes had the highest expression, followed by spleen, lung and heart. Both variants were expressed at similar levels, and were almost equally induced by DNA synthesis inhibitors (hydroxyurea and cytosine arabinoside) or a differentiation inducer (all-trans retinoic acid) in HL-60 cells.

3.3. Differences in ligand-evoked responses between G2A-a and G2A-b

Since G2A-a and G2A-b have slight differences in the N-terminal region, we compared ligand-evoked intracellular responses between these variants. As for 9-HODE, there was no obvious difference in intracellular calcium mobilization or ligand-induced GTPγS binding based on the 9-HODE concentrations required for inducing the half-maximum response. On the other hand, G2A-b showed slightly higher IP accumulation than G2A-a at every pH examined ranging from 6.2 to 8.2, although the overall tendencies were similar. Taken together, G2A-a and G2A-b are similarly expressed, induced and responsive to ligand stimulation, although they are slightly different in terms of proton-sensing activity.

4. Physiological functions of G2A as a receptor for oxidized free fatty acids

G2A deficient mice are susceptible to a late-onset autoimmune syndrome that is due to defects in lymphocyte selection [13]. This has been partly explained in reports showing that G2A is involved in trafficking of lymphocytes and monocytes [35,36]. The involvement of G2A in atherosclerosis was also reported. G2A is highly expressed in macrophages deposited in atherosclerotic plaques both in human and in apolipoprotein E deficient mice [37]. Loss of G2A attenuated the progression of atherosclerotic lesions in LDL receptor deficient mice [15]. However, these observations in mice should be carefully interpolated to G2A functions in human, because mouse G2A does not respond to 9-HODE as mentioned above.

In addition to 9-HODE, human G2A can respond to various types of H(P)ODEs and HETEs without strict chiral selectivity. Although the 9-HODE concentration required to induce the half maximum response in the intracellular calcium assay (∼0.5 μM) is relatively high for a lipid mediator, it could be achieved as a mixture of oxidized linoleic and arachidonic acids where massive oxidative stress occurs.

4.1. Role of G2A in keratinocyte

Linoleic acid is abundantly contained in the epidermis and is essential for proper cutaneous barrier function. Linoleic acid-rich acylceramide is thought to play an important role in the formation of stratum corneum lipid layers [38]. In addition to ceramides, cholesteryl ester and free fatty acids are abundant in the stratum corneum, and linoleic acid is the most abundant species of polyunsaturated fatty acid both in esterified forms and as free fatty acids [39]. In this context, when skin is exposed to oxidative stress such as UV irradiation and microorganism infection, 9-HODE converted from free linoleic acid in the stratum corneum might have some effects on the keratinocyte layers beneath.

4.1.1. Cellular effects of 9-HODE on keratinocytes

In normal human epidermal keratinocytes (NHK cells), 9-HODE suppressed cell proliferation. 9-HODE almost completely blocked proliferation at the concentration of 10 μM. Cell cycle analysis revealed that cell accumulation occurred at the G1 phase in 24 h after stimulation. Apoptosis was not observed at this time point. In addition to growth suppression, 9-HODE induced prominent morphological changes in NHK cells; the cells overlaid each other and contained enlarged cytoplasm with distinct shiny perinuclear vesicles.

Of 18 cytokines examined, 9-HODE induced the secretion of interleukin (IL)-6, IL-8 and GM-CSF in NHK cells. The time course of the secretion differed slightly between the three cytokines. The secretion of IL-6 occurred earliest, observed as early as at 4 h after the stimulation. Secretion of IL-8 and GM-CSF was evident after 16 h incubation.

4.1.2. Involvement of G2A in 9-HODE-evoked keratinocyte responses

Immunohistochemical analysis revealed that G2A was preferentially expressed in the spinous and granular cell layers in human shoulder skin sections. G2A expression was also observed in NHK

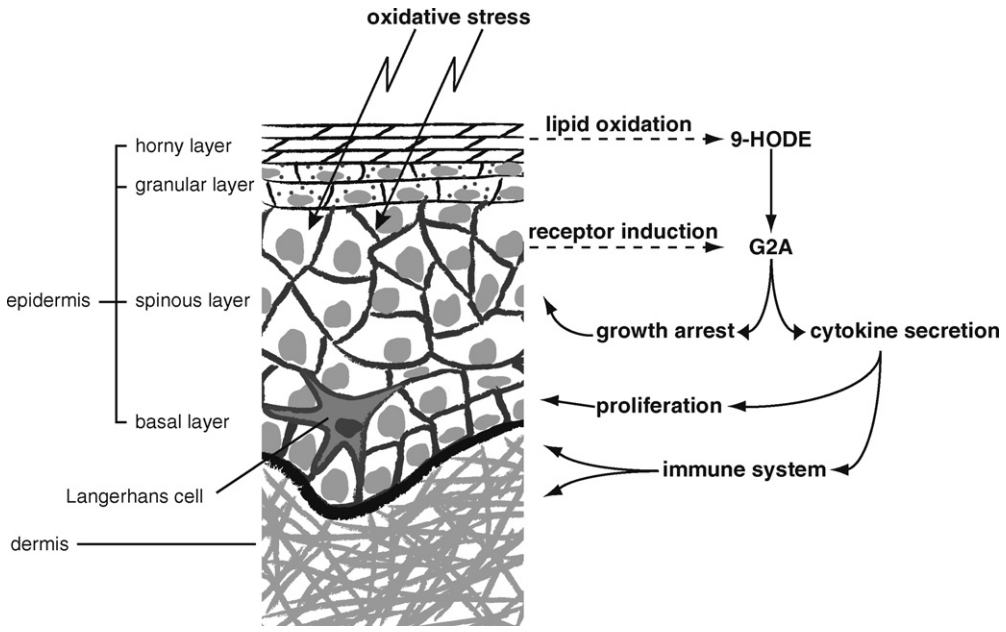

Fig. 2. Putative functions of G2A in the epidermis. Oxidative stress induces 9-HODE generation from linoleic acid that is abundantly contained in the corneum stratum. Then, 9-HODE acts on G2A that is also induced by oxidative stress in the granular and spinous layer. G2A mediates cell cycle attenuation and cytokine secretion. Secreted cytokines promote proliferation of keratinocytes in the basal layer or activate immune system.

cells and in HaCaT cells (an immortalized human keratinocyte cell line).

In contrast to 9-HODE, 13-HODE had no or much smaller cellular effects on NHK cells. Moreover, siRNA, which was designed to suppress G2A expression, partially blocked 9-HODE-induced growth arrest. When G2A was overexpressed in HaCaT cells, the cytokine secretion by 9-HODE was markedly increased. These results indicated that the effects of 9-HODE are mediated by G2A. PPARγ is known to work as a receptor for oxidized free fatty acids including 9-HODE and 13-HODE in macrophages [40]. However, it is unlikely that 9-HODE-induced cellular effects are mediated via PPARγ in keratinocytes, because PPARγ does not discriminate between 9- and 13-HODE, and a much higher concentration (over 30 μM) is required to activate PPARγ.

4.1.3. Induction of G2A and generation of 9-HODE by oxidative stress

G2A induction was observed during oxidative stress such as UV irradiation and H_2O_2 stimulation in HaCaT cells. When the cells were subsequently stimulated with 9-HODE, cytokine secretion was also enhanced. These results suggest that oxidative stress induces functional G2A, and enhances cellular responses to 9-HODE.

In addition to G2A induction, oxidative stress induced the conversion of 9-HODE from linoleic acid. When HaCaT cells were exposed to UVB or H_2O_2, substantial generation of 9- and 13-HODE was observed in a time- and dose-dependent manner. The UVB dose required for the significant increase was 25 mJ/cm^2 that is in the range we are commonly exposed to in daily life. Thus oxidative stress induces G2A expression in the epidermis on the one hand, and 9-HODE generation on the other hand from linoleic acid that is abundant in the uppermost corneum stratum.

4.1.4. Putative physiological functions of G2A in keratinocytes

9-HODE works on G2A to suppress cell proliferation and induce cytokine secretion such as IL-6 and IL-8. These actions seem to be appropriate cellular responses when cells are damaged by oxidative stress. The damaged keratinocytes should then halt their proliferation or differentiation. On the other hand, healthy keratinocytes in the basal layer should start differentiation to replace the damaged ones. Both IL-6 and IL-8 are known to stimulate the proliferation of keratinocytes [41,42]. Thus G2A might be involved in maintaining appropriate turnover of keratinocytes under oxidative conditions.

On the other hand, 9-HODE was reported to be a strong pro-inflammatory mediator in an experimental wound-healing model in rats [43]. IL-8 is a chemokine that recruits neutrophils, macrophages and T cells to evoke inflammation. The abnormally high levels of IL-6 and IL-8 would be associated with the hyperproliferation of keratinocytes and the accumulation of neutrophils, which are characteristic findings of psoriatic lesions [41,44]. G2A might be involved in the initiation of various pathological conditions such as inflammation and psoriasis through cytokine secretion from keratinocytes. Fig. 2 summarizes the putative functions of G2A in the epidermis.

4.2. Cell cycle regulation by G2A

G2A was originally identified as a stress inducible receptor that blocks cells at the G2/M phase in NIH 3T3 cells [1]. The accumulation of cells at the G2/M phase was ligand-independent and induced just by overexpressing G2A. p53 was not required for the G2/M block or for the induction of G2A by analysis of fibroblasts isolated from p53 null mice [1].

In keratinocytes, merely overexpressing G2A did not have obvious effects on cell proliferation. Instead, cell proliferation was blocked at the G1 phase in a 9-HODE-dependent manner. When the expression of cell cycle-related genes was analyzed in NHK cells, cyclin D protein was decreased within 4 h after 9-HODE stimulation. At the same time, $p15^{INK4B}$, a CDK inhibitor, was increased (unpublished observation). We assume that the increase in $p15^{INK4B}$ leads to down regulation of cyclin D and subsequent cell cycle arrest, but the precise mechanism remains to be clarified. While there are numerous studies on proliferative regulation by GPCRs mostly via MAPK activation, anti-proliferative regulation by GPCRs is not well understood. It would be considerable interest to elucidate how G2A blocks cell proliferation and what the differences are between G1 and G2/M accumulation.

5. Concluding comments

We assume that G2A functions in vivo as a sensor of oxidative stress based on the analysis of keratinocytes. Oxidative stress can induce both the expression of G2A and the production of its ligands, which leads to the anti-proliferative responses in damaged cells, and to the production of paracrine signals to evoke proliferation or inflammation in neighboring cells. In this sense, it is an attractive subject to explore the involvement of G2A in atherosclerosis. Oxidized LDL plays important roles both in triggering and in progression of atherosclerotic lesions that are formed by complex interactions of macrophages, lymphocytes and vascular endothelial cells. It has been reported that G2A is involved in the regulation of monocyte/endothelial interactions in the aorta [45]. We are now examining the effects of 9-HODE on these cells.

One of the other important issues will be to clarify the role of mouse G2A. Does it have a distinct role from human G2A as a receptor for unidentified ligands? Or does it play roles equivalent to human G2A without ligands? The answers to these questions would be help understand the phenotypes observed in G2A knockout mice such as the late-onset autoimmune syndrome.

As far as we know, G2A is the first GPCR that recognizes non-enzymatically oxidized free fatty acids. In addition to linoleic and arachidonic acid, there are several other polyunsaturated fatty acids found in vivo such as linolenic, eicosapentaenoic and docosahexaenoic acid. A variety of oxidized fatty acids can be produced from these lipids during oxidative stress, and might function as signaling molecules. It is possible that some orphan GPCRs mediate these signals. Comprehensive analysis of oxidized fatty acids by mass spectrometry will help us to identify the novel lipid mediator(s), and to understand the roles of polyunsaturated fatty acids beyond their function as structural components.

Acknowledgements

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