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Resolution of Inflammation by *N*-Arachidonoylglycine

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ABSTRACT

N-arachidonoylglycine (NAgly) is an endogenous signaling lipid that is a member of the eicosanoid super family and is related to anandamide. It shows anti-inflammatory activity in vivo in the mouse peritonitis model where it reduces migration of inflammatory leukocytes following injection of pro-inflammatory agents into the peritoneal cavity. Using cell culture models, including GPR18 transfected HEK-293 cells, evidence is presented that the orphan receptor GPR18 is involved in this action. Increases in free arachidonic acid, and robust stimulation of anti-inflammatory eicosanoids were observed at low micromolar concentrations. These included 15-deoxy-delta-13,14-PGJ_2 and lipoxin A_4 both of which are believed to mediate the resolution stage of inflammation. It was further shown that NAgly might act via GPR18 activation in promoting the number of Trypan Blue stained cells, a possible indicator of programmed cell death. Thus, we hypothesize that NAgly induces the death of inflammatory cells, a process that is considered to be important for the resolution of inflammation. J. Cell. Biochem. 112: 3227–3233, 2011.

KEY WORDS: INFLAMMATION; *N*-ARACHIDONOYLGLYCINE; GPR18; ANTI-INFLAMMATORY EICOSANOIDS; CELL DEATH; qPCR

Inflammation is a primitive response that protects against injury and infection with the ultimate aim of restoring damaged tissue to its normal physiological state, and to which we are alerted by pain, swelling, and redness [Ryan and Majno, 1977]. Defects in the response can redirect physiological resolution towards inflammation. It is well established that specific eicosanoids, lipid mediators derived from arachidonic acid by the actions of cyclooxygenases (COXs) and lipoxygenases (mainly prostaglandin E_2 and leukotriene B_4), contribute to the initiation of inflammation [Samuelsson, 1991]. In addition, the process is driven by activation of inflammatory cells (mainly polymorphonuclear leucocytes and monocyte/macrophages), which release inflammatory cytokines, mainly IL-1 beta, IL-6, and tumor necrosis factor alpha (TNFα).

It was long thought that resolution of inflammation was a passive process. Contrary to this belief, the inflammatory response does not dissipate spontaneously, but is mediated by other metabolites of arachidonic acid [Stables and Gilroy, 2010]. Gilroy et al. [1999] observed that blockade of COX activity in a murine model of peritonitis results in suppression of acute inflammation but leads to perpetuation of inflammation by interfering with its resolution. The concentration of PGJ (15-deoxy-delta-13,14-PGJ_2) formed from the bioconversion of PGD_2 increases during the resolution phase, and acts as a brake on inflammation by, among other actions, inducing apoptosis of inflammatory cells [Herlong and Scott, 2006]. The concentration of the lipoxygenase product LXA_4 (lipoxin A_4) is also increased during the resolution phase and acts as a stop signal for the acute response. In addition, programmed cell death (PCD) of inflammatory cells is an important part of the resolution process [Bannenberg and Serhan, 2010]. For example, inflammatory and synovial cells from joints of patients with rheumatoid arthritis are resistant to apoptosis, which interferes with the resolution of inflammation and leads to chronic inflammation and joint tissue injury.

The data reported here indicate that NAgly (*N*-arachdonoylglycine), a member of the eicosanoid superfamily, has the ability to increase production of PGJ and LXA_4, reduce migration of inflammatory cells into an area of acute inflammation and induce the death of inflammatory cells. Thus, NAgly should have a physiological role in the resolution of an acute inflammatory response and, in addition, be a potential candidate for the therapy of

Abbreviations used: COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; LXA_4, lipoxin A_4; NAgly, *N*-arachidonoylglycine; NRS, normal rabbit serum; PALgly, *N*-palmitoylglycine; PGJ, 15-deoxy-delta-13,14-prostaglandin J_2; PCD, programmed cell death; TNFα, tumor necrosis factor alpha.

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conditions characterized by ongoing (chronic) inflammation. Our data suggest that this action of NAgly is mediated by anti-inflammatory, pro-resolving eicosanoids and the G-protein coupled receptor GPR18.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

NAgly and PALgly (*N*-palmitoylglycine) were obtained from Dr. Robert Zipkin, (Medchem 101, Conshohocken, PA). GPR18 antibody was purchased from Assay Biotechnology Company, Inc. (Sunnyvale, CA); the immunogen was the amino acid sequence 151–180. This immunizing sequence corresponds to most of the extracellular loop 2 (between TM4 and TM5). The IgG fraction of normal rabbit serum was purchased from (Sigma–Aldrich, St. Louis, MO).

HEK293/GPR18 TRANSFECTED CELLS

Human embryonic kidney cells (HEK293) transfected with GPR18 were generously supplied by Dr. Heather Bradshaw; these were reported to be highly responsive to NAgly in a cell migration assay [McHugh et al., 2010]. The transfection and validation procedures are fully described by them.

CELL CULTURE

HEK293 cells were maintained in RPMI medium containing 10% FBS and TNFα (10 ng/ml final conc.) and incubated at 37°C/5% CO_2. U937, HL60, MOLT4, and RAJI cells were purchased from ATCC (Manassas, VA, 20108) and cultured using their suggested conditions.

STIMULATION OF ARACHIDONIC ACID RELEASE BY NAGLY

C6 glioma cells (ATCC) were grown and treated as described previously [Pestonjamasp and Burstein, 1998]. Following a 2 h labeling period with ^{14}C-arachidonic acid, the media were changed to serum-free RPMI + 0.1% BSA and the cells treated for with NAgly in 10 μl of DMSO. The control was 10 μl of DMSO. N = 4. Release was measured by liquid scintillation counting on a 0.1-ml aliquot of medium. Values shown are the mean ± SD. *Note*: The cells were treated with nonradioactive NAgly.

EICOSANOID MEASUREMENTS

Elisa assay kits for PGJ and LXA_4 were obtained from Assay Designs, Inc. (Ann Arbor, MI). The identity of the PGJ analyte in the culture medium was confirmed by mass spectrometry (Wood and Makriyannis, unpublished data). Treatments were carried out in 48 well plates with 50,000 cells/500 μl DMEM + FCS media/well and TNFα (10 nM) added. Cells were incubated for 20 h at 37°C and 5% CO_2. Media were changed to 500 μl of serum-free DMEM, treated for 2 h and 100 μl removed for assays. Assays were carried out with PGJ or LXA_4 ELISA kits (Assay Design, Inc.). Control: 1% DMSO vehicle. N = 4.

PERITONITIS ASSAY

This study was carried out by BRM, Inc. (Worcester, MA). NAgly and PALgly were administered p.o. in 50 μl of safflower oil; after 30 min, the mice were injected i.p. with 1 ml (sterile filtered) 8% BBL Fluid Thioglycollate Medium (Becton Dickinson). Cells were harvested after 3 h, exposed to lysing buffer for 2 min to remove erythrocytes, suspended in PBS/BSA and an aliquot assayed for cell numbers by the Titer Glo assay (Promega). The remaining cells were subjected to a differential cell count. N = 4. All animal studies were performed according to institutional, local, state, federal, and NIH guidelines for the use of animals in research under an Institutional Animal Use and Care Committee (IACUC)-approved protocol.

QUANTITATIVE REAL-TIME PCR (qPCR) ANALYSIS

Total RNAs were extracted from the various cell lines using TRIZOL reagent (Invitrogen). The cDNAs were prepared with a SuperScript II Cells Direct cDNA synthesis system (Invitrogen). The expression of the GPR18 gene was quantified using a QuantiTect SYBR Green PCR kit (Qiagen) in an MJ Research DNA Engine OPTICON (BioRad), and corrected with a hypoxanthine phosphoribosyl transferase 1 (HPRT1) control. Amplifications were done in a total volume of 25 μl for 45 cycles of 15 s at 95°C and 1 min at 60°C. The relative expression was determined by normalizing the expression of each target to HPRT1 and then comparing this normalized value with the normalized expression in a reference control sample to calculate the fold change value (ΔCt). The primers for the amplicons will span intron/exon boundaries to minimize amplification of genomic DNA. Primer sequences are as follows:

GPR18, forward 50-TTCTTGATCTGCTGACCATGACAC-30; reverse 50-AGGGACAGGTTGATCTTGATTGTTC-30;
HPRT1, forward 50-TAAGCCAGACTTTGTTGGAT-30; reverse 50-GAACTCTCATCTTAGGCTTT-30.

ANNEXIN STAINING

Two T-75 flasks were seeded with 100,000 HEK293 GPR18 transfected cells/ml, TNFα (10 ng/ml final conc.) was then added in DMEM + 10% FBS and the cells were incubated overnight. Cells then treated with 5 μM NAgly in DMSO (1% final concentration) or DMSO only, and incubated at 37°C and 5% CO_2 for 2.5 h. Cells were then detached by scraping, dispersed by mild agitation and divided into 12 tubes/group, stained according to the protocol for BD Pharmingen FITC Annexin V Apoptosis Detection Kit II (Cat. # 556570) and analyzed by FACS. N = 3.

RESULTS

MOUSE PERITONITIS ASSAY

The data shown in Figure 1 suggest an inverse dose response relationship for the inhibition of thioglycollate-induced inflammation and migration of cells into the peritoneal cavity by NAgly. At doses of 0.3 and 1.2 mg/kg given orally (not gavage) a greater than 50%, statistically significant, inhibition of peritoneal cell content was observed using a two tailed *t*-test and Prizm software (GraphPad). Increasing the dose to 20 mg/kg returned the response to vehicle control levels. Differential cell analysis indicated that the cells consisted primarily of monocytes and neutrophils in a roughly 2:1 ratio for all treatment groups. By comparison, PALgly, a

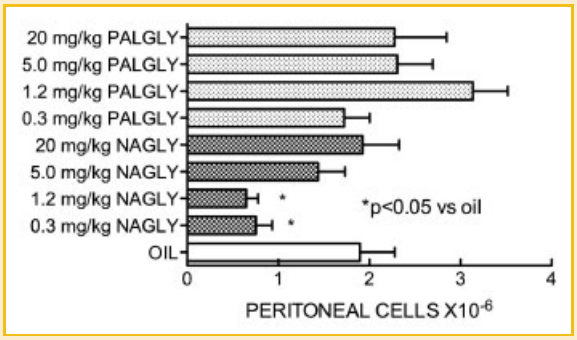

Fig. 1. NAgly inhibits leukocyte migration in the mouse peritonitis model. The indicated treatments were administered by mouth and after 30 min, the mice were injected i.p. with 1 ml (sterile filtered) 8% BBL Fluid Thioglycollate Medium. Cells were harvested from the peritoneal cavity after 3 h, exposed to lysing buffer for 2 min to remove erythrocytes, suspended in PBS/BSA and differential cell counts obtained. Controls were safflower oil and Palmitoylglycine (PALgly). N = 8.

negative control, had no significant effect on cell migration over the same dose range used for NAgly.

INCREASED FREE RADIO-LABELED ARACHIDONIC ACID CONCENTRATIONS

A robust release of free arachidonic acid from cells whose storage sites contained radio-labeled arachidonate was observed following treatment with unlabelled NAgly (Fig. 2). The effect was both time and concentration dependent. The latter suggests a possible bell-shaped response with a maximum around 6 μM.

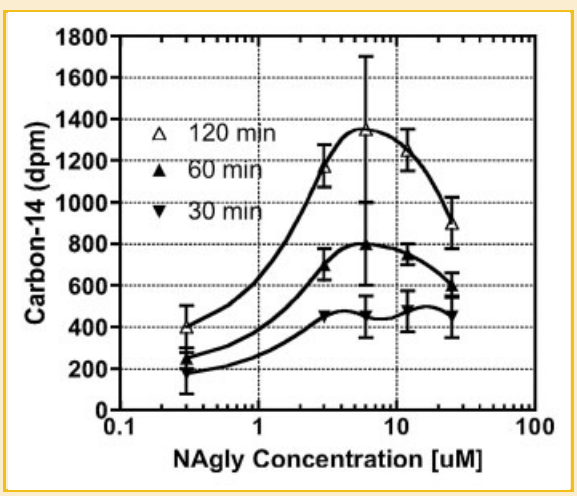

Fig. 2. Stimulation of arachidonic acid release from C6 glioma cells by NAgly. Cells were grown and treated as described previously [Pestonjamasp and Burstein, 1998]. See also "Materials and Methods" Section. Following a 2-h labeling period with ^{14}C-arachidonic acid, the media were changed to RPMI + 0.1% BSA and the cells treated with NAgly. Release into the media was measured by LSC of aliquots of medium. Values shown are the mean ∓ SD. N = 4. Control, DMSO treated cells gave values of 190, 250, and 385 dpm, respectively. Note: The cells were treated with nonradioactive NAgly.

STIMULATION BY NAgly OF THE LEVELS OF PGJ AND LXA_4

Treatment of transfected HEK293 cells with NAgly produced a robust increase in the levels of PGJ (Fig. 3A) and LXA_4 (Fig. 3B). NAgly appeared to be more effective in stimulating PGJ; both results were measured in the same experiment. In a similar study, treatment of four cell human cell lines, U937, HL60, MOLT4, and RAJI, produced a range of PGJ responses (Fig. 4) that were compared with GPR18 mRNA levels (see below). There was a statistically significant correlation between GPR18 mRNA levels and PGJ stimulation In these same cells, prior treatment with the CB1 antagonist SR141716 (10 μM) or the CB2 antagonist SR144528 (1 μM) had no effect on the 3 μM NAgly induced rise in PGJ or LXA_4 (unpublished).

GPR18-MEDIATED PATHWAY

Support for a role for GPR18-mediated PGJ synthesis was sought by the use of a GPR18 antibody as shown in Table I. The immunizing sequence for the production of the antibody corresponds to most of the extracellular loop 2 (between TM4 and TM5). Treatment of transfected HEK293 cells with 5 μM NAgly caused a 40-fold increase of PGJ concentrations in the media. The addition of the polyclonal anti-GPR18 reduced this response to a fourfold increase suggesting that GPR18 mediates this action. A control study was also carried out using the crude IgG fraction from normal rabbit serum (NRS) (Table I). In this experiment a 37-fold stimulation of PGJ following 5 μM NAgly was seen, which was only somewhat reduced to 26.3-fold in the presence of NRS IgG.

EFFECTS ON CELL SURVIVAL

Table II contains data showing that 3 μM NAgly treatment of U-937 cells causes a 2.14-fold increase in Trypan Blue staining that is mimicked by PGJ at a 4-nM concentration; the latter is in the range of PGJ production in our cell culture model (Fig. 3). Table III shows a similar effect of NAgly on staining when HEK293 GPR18 transfected cells are used. In addition, data are presented showing that pretreatment with anti-GPR18 completely blocks the NAgly-induced increase in Trypan Blue stained cells. A control treatment with NRS IgG did not prevent the NAgly-induced increase in Trypan Blue staining.

Figure 5 shows by FACS analysis the effect of 5 μM NAgly on staining with propidium iodide and annexin V-FITC when HEK293 GPR18 transfected cells are used. Panels A and B are representative examples from triplicate measurements following treatment for 2.5 h with NAgly and vehicle control. The results obtained for the upper two quadrants are of interest where late-stage apoptotic cells (upper right) and necrotic cells (upper left) are shown. Panel C shows the mean fluorescence values obtained for the triplicate values and indicates a decrease in late-stage apoptotic cells and an increase in necrotic cells as a result of drug treatment.

DISCUSSION

NAgly is the most important member of a subfamily of lipoamino acids called elmiric acids that was first described in 1997 [Burstein et al., 1997; Burstein, 2008]. It is an endogenous substance in rat tissues and may play a role in the regulation of pain and

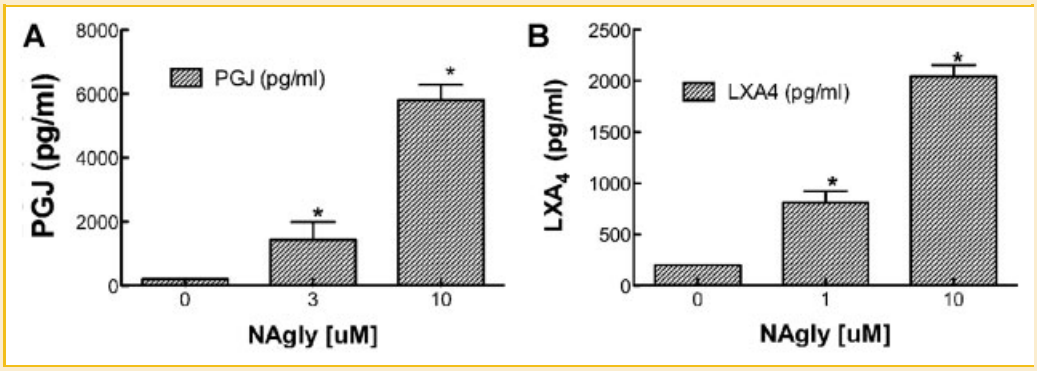

Fig. 3. Stimulation by NAgly of levels of PGJ and LXA_4 in HEK393 GPR18 transfected cells. Treatments were carried out in 48-well plates with 20,000 cells/0.5 ml RPMI/FCS media/well. Cells were incubated for 20 h at 37°C and 5% CO_2. Media were changed to 0.5 ml of serum free RPMI and TNFα (10 nM) added, treated for 2 h and 0.1 ml removed for the assays. Assays were performed with PGJ or LXA_4 ELISA kits (Assay Design, Inc.). Controls: 1% DMSO vehicle. N = 4.

inflammation responses. Huang et al. [2001] also reported the endogenous existence of NAgly and two of its congeners suggesting the existence of a family of lipoamino acids. In fact, a recent review indicates that there are at least 50 naturally occurring members of the lipoamino acids [Bradshaw et al., 2009]. The origin of endogenous NAgly has been studied and it was concluded that two synthetic pathways exist [Bradshaw et al., 2009]. It can arise either by a direct reaction between glycine and arachidonic acid, or, from the oxidation of anandamide, which is an alcohol precursor and a CB1 receptor ligand. In addition to its analgesic properties [Vuong et al., 2008; Barbara et al., 2009], NAgly shows anti-inflammatory effects [Burstein et al., 2007], vasorelaxant properties [Parmar and Ho, 2010], effects on cell migration [McHugh et al., 2010], reduction of calcium channel function [Ross et al., 2009], and inhibition of fatty acid amide hydrolase (FAAH) [Cascio et al., 2004] the anandamide inactivating enzyme.

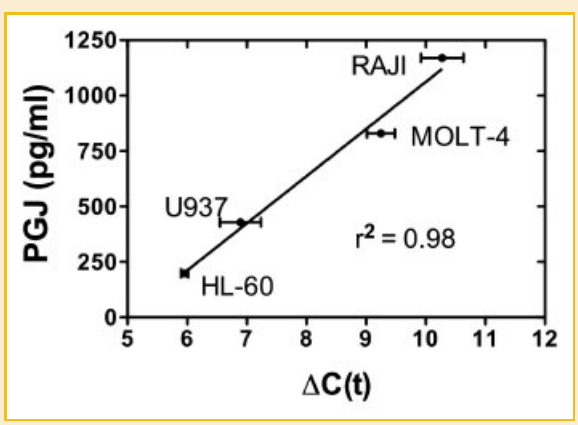

Fig. 4. Correlation of GPR18 mRNA levels with PGJ stimulation by NAgly. See "Materials and Methods" Section for the procedures used. For the relative comparison of mRNA expression levels, the data from real-time PCR were analyzed with a delta–delta Ct method and normalized to the amount of HRPT1 cDNA as an endogenous control. Controls for PGJ assays were 1% DMSO vehicle. N = 3.

Inhibition of cell migration into the mouse peritoneal cavity following injection of a pro-inflammatory substance such as thyioglycollate is an established method for measuring anti-inflammatory action of a drug. Earlier preliminary findings suggested that NAgly would produce such a response [Burstein and Zurier, 2009]. The data shown in Figure 1 confirms and extends this finding of in vivo activity to reveal a complex dose relationship. The earlier report suggested a "bell-shaped" dose response and further implicated possible involvement of PGJ [Burstein and Zurier, 2009], an anti-inflammatory eicosanoid [Stables and Gilroy, 2010]. Figure 1 also shows no anti-inflammatory activity for the analog PALgly (palmitoylglycine) indicating the existence of structural requirements for this type of action. These observations raise the possibility of receptor involvement and further suggest that this is a site of action for the resolution stage of inflammation. PGJ and LXA_4 are bioactive lipids resulting from the actions of COXs and lipoxygenases [Serhan, 2010] on free arachidonic acid. Serhan has suggested that lipoxins, whose synthesis is mediated by lipoxygenases, can promote the resolution of inflammation.

NAgly does not bind to a CB1 preparation [Sheskin et al., 1997], which is in agreement with its low response [Burstein et al., 1997] in

TABLE I. Antibody Inhibition of NAgly-Stimulated PGJ Synthesis in HEK293 GPR18 Transfected Cells

Rx1 (15 min)	Rx2 (3 h)	PGJ (pg/ml)	Treated/control
PBS	DMSO	50.0	1
PBS	NAgly [5 μM]	2,012	40.2*
Anti-GPR18	DMSO	707	1
Anti-GPR18	NAgly [5 μM]	2,799	3.96**
PBS	DMSO	90.0	–
PBS	NAgly [5 μM]	3,325	36.9*
NRS IgG	DMSO	306	–
NRS IgG	NAgly [5 μM]	8,085	26.3***

Treatments were carried out as described in "Materials and Methods" Section entitled Eicosanoid measurements. In a control study, normal rabbit IgG did not inhibit the NAgly stimulatory effect. PBS, phosphate buffered saline.
*$P < 0.004$ versus PBS/DMSO control.
**$P < 0.006$ versus anti-GPR18/DMSO control.
***$P < 0.006$ versus NRS IgG/DMSO control.

TABLE II. PGJ Induction of Trypan Blue Staining in U-937 Cells (Stained Cells/Total Cells)

Rx (3 h)	% TB stained	Treated/control[a]
DMSO	22	1.00
3 μM NAGLY	47**	2.14
4.0 nM PGJ	33**	1.50

Cells treated as described in "Materials and Methods" Section entitled Eicosanoid measurements.
Controls: 1% DMSO treated cells; 3 μM NAgly. N = 3.
[a]Trypan Blue stained cells/DMSO control.
**$P < 0.05$ versus DMSO

the "ring test" for cannabimimetic activity [Pertwee, 1972]. This test involves a cataleptic effect in mice generally believed to be mediated by CB1. These observations are remarkable because NAgly differs from anandamide, a CB1 agonist, by only a single oxygen atom. Using the technique of degenerate-oligonucleotide PCR analysis, a putative receptor for NAgly was recently identified as the orphan receptor GPR18 [Kohno et al., 2006]. A library of 198 bioactive lipids was screened for activation of GPR18 transfected cells with calcium ion mobilization as the indicator. Only NAgly gave a significant response suggesting a high degree of structural specificity for this receptor. In GPR18-transfected CHO cells, NAGly inhibited forskolin-induced cAMP production in a manner typical for a G-protein coupled receptor. Until now, other than the report of McHugh et al. [2010] on directed cell migration, little else has been published on the molecular events responsible for the effects of NAgly.

One of the actions of NAgly that we are especially interested in is its apparent promotion of the resolution phase of inflammation. Earlier studies in mice suggest that NAgly and some of its analogs may provide a useful template for the design of novel anti-inflammatory drugs [Burstein et al., 2007]. Of interest was the observation that there is a good correlation between in vivo action and in vitro responses in a cell culture model in which stimulation of specific eicosanoid production is the marker [Burstein, 2008]. Thus, our hypothesis is that NAgly, by activating GPR18, increases the levels of anti-inflammatory eicosanoids such as PGJ and/or LXA_4, leading to the resolution of inflammation. Based on this hypothesis, we now describe several molecular events initiated by the interaction of NAgly with GPR18 that supports this hypothesis.

The release of free arachidonic acid is the rate-limiting step in most eicosanoid-mediated pathways. Treatment of C6 glial cells in the presence of BSA with NAgly resulted in a rapid and robust release of free arachidonic acid (Fig. 2) confirming an earlier similar observation in RAW cells [Burstein, 2008]. The BSA limits the re-esterification of arachidonic acid. In this study, esterified storage sites were radio-labeled with C^{14}-arachidonic acid and the release of radioactivity, following NAgly exposure, into the media was measured. The free acid came from storage sites in the cells and not from the added NAgly, which was not radio-labeled. The released radioactivity may also contain variable amounts of eicosanoids, which are all metabolites of arising from the free arachidonic acid.

Unless a fatty acid trapping agent such as BSA is present, most cell culture models readily convert released arachidonic acid to one or more members of the eicosanoid super family. Such was the case in our HEK293 cell model where robust increases in both PGJ and LXA_4 were observed (Fig. 3). Earlier studies with NAgly [Burstein, 2008] indicated that relatively small amounts of PGE were produced compared with the increase in PGJ that was generated by NAgly treatment. The reason for this specificity in biosynthesis is not known at this time. Nevertheless, this effect could be a major factor in the anti-inflammatory actions of NAgly. The data shown in Table I suggest that this effect may be mediated by the recently de-orphanized receptor GPR18. When compared to the appropriate control, NAgly-induced PGJ synthesis is decreased from 40-fold to approximately 4-fold. Table I also shows data from a second experiment where NRS IgG was substituted for anti-GPR18. This procedure resulted in a much smaller inhibition of the NAgly effect supporting our hypothesis that GPR18 is a mediator in this response. A complicating factor in the use of anti-GPR18 is its apparent agonist activity on PGJ production (Table I) and Trypan Blue staining (Table III). In spite of this anti-GPR18-mediated agonist activity, which may involve some other constituent in the antibody preparation, significant effects of NAgly-mediated action were readily demonstrated in our models.

Recent reports provide supporting evidence that GPR18 is a target for NAgly [Kohno et al., 2006; McHugh et al., 2010]. We have examined the possibility that this may be an important component in the signaling pathway for the expression of the anti-inflammatory actions of NAgly. The findings presented here using real-time PCR are in agreement with this idea (Fig. 4). We have been able to show an excellent correlation between the PGJ response and qPCR data from four human cell lines, RAJI, U-937, HL-60, and MOLT-4, each with distinct expression levels of GPR18 as reported by Kohno et al. [2006] and confirmed by us. These data infer that some type of action mediated by GPR18 regulates the production of PGJ.

A possible indicator of drug-induced cell death is the observed increase in the percentage of cells that are stained with Trypan Blue following exposure to 3–5 μM NAgly (Tables II and III). Of particular note is the observation that 4 nM PGJ also is able to cause a similar response. This remarkable finding supports the idea that PGJ is a mediator in this action of NAgly consistent with our proposed

TABLE III. Trypan Blue Staining of HEK293 GPR18 Transfected Cells

Rx1 (15 min)	Rx (3 h)	% TB stained	Treated/control[a]
PBS	DMSO	4.1	1
PBS	NAGLY [5]	7.3	1.78***
NRS IgG[b]	DMSO	4.4	1
NRS IgG[b]	NAGLY [5]	12.4	2.82***
Anti-GPR18	DMSO	10.5	1
Anti-GPR18	NAGLY [5]	10.4	0.99***

Treatments carried out as described in "Materials and Methods" Section entitled Eicosanoid measurements.
[a]Trypan Blue stained cells/DMSO control.
[b]Same concentration as anti-GPR18 (NRS, normal rabbit serum).
***$P < 0.05$ versus corresponding DMSO control by t-test analysis.

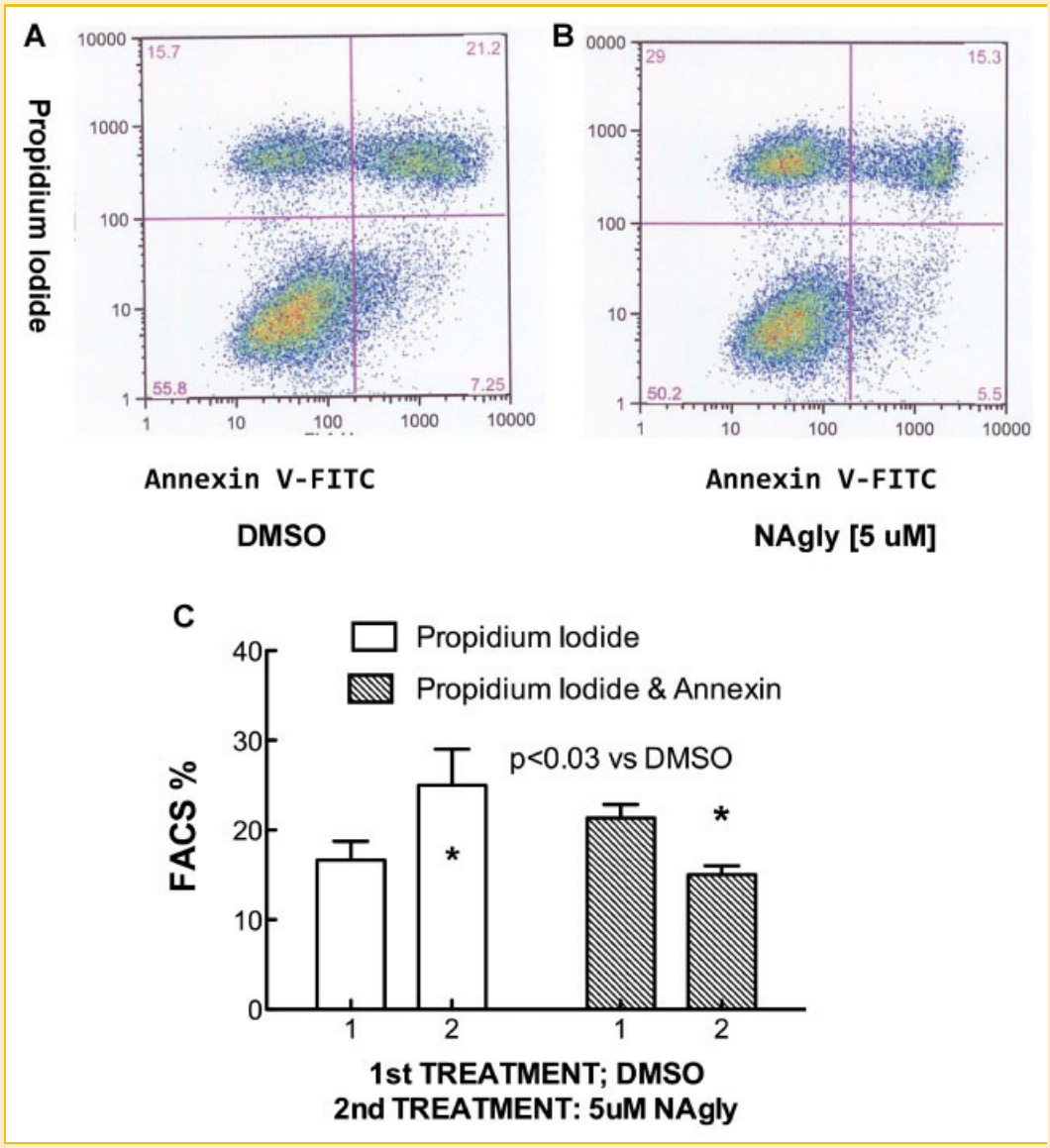

Fig. 5. FACS analysis following NAgly treatment of HEK293 GPR18 transfected cells. Two T-75 flasks were seeded with 100,000 HEK293 GPR18 transfected cells/ml, TNFa (10 ng/ml final conc.) was added to the cells in DMEM + 10% FBS and incubated overnight. Cells were then treated with 5 μM NAGLY in DMSO (A) or DMSO only (B), and incubated at 37°C and 5% CO_2 for 2.5 h. Cells from each flask were divided into 12 tubes/group, stained according to the protocol for BD Pharmingen FITC Annexin V Apoptosis Detection Kit II (Cat. # 556570) and analyzed by FACS. Panels A and B are representative data. Panel C shows the mean fluorescence values obtained for late-stage apoptotic cells (upper right) and necrotic cells (upper left). N = 3. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

mechanism for anti-inflammatory action. Moreover, the use of anti-GPR18 to block the effect of NAgly on the Trypan Blue response in transfected HEK293 cells implicates the GPR18 receptor in some form of cell death (Table III). A control experiment with NRS IgG did not block the anti-GPR18 effect suggesting that the latter is due to a specific interaction with the receptor.

A FACS analysis following annexin staining of HEK293 GPR18 transfected cells supports the possible occurrence of PCD following treatment for 2.5 h with 5 μM NAgly (Fig. 5). Panels A and B show examples of the readouts from this analysis of vehicle and drug treated cells, respectively. Panel C summarizes these findings and shows a greater than 50% increase in cell death together with a comparable decrease in late-stage apoptosis suggesting a temporal connection between these two events.

Taken together, all of the data presented suggest a mechanism for the anti-inflammatory action of NAgly as follows. NAgly activates GPR18 causing a release of free arachidonic acid from its esterified cellular storage sites. In turn, this enters the arachidonic acid cascade resulting in an elevation of the anti-inflammatory eicosanoids PGJ and LXA_4. An increase in cell death then ultimately occurs, presumably directed at pro-inflammatory cells and leading to the resolution of inflammation. In vivo this manifests itself as a

decrease in the migration of cells to the site of inflammation. Such a scheme represents only the outline of the entire mechanism and many of the intermediary steps still remain to be elucidated.

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