3-Hexanoyl-7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol (3-NBD-cholesterol) is a versatile cholesterol tracer

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ABSTRACT
Membrane cholesterol influences a large number of cellular processes and the dynamics of cholesterol exchange between membranes is an area of active study. However, analogs containing a fluorophore on the isooctyl side chain of cholesterol are commonly used without regard for the potential impact of the fluorophore on membrane structure. We investigated the capacity of 3-hexanoyl-7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol (3-NBD-cholesterol), which is labelled at the C3 position, to trace cholesterol dynamics in cellular systems. Transfer of 3-NBD-cholesterol from erythrocytes to lipoproteins replicated known properties of cholesterol. Labelled cells were also readily detected by flow-cytometry and microscopy. Using flow-cytometry it was also possible to follow the uptake of 3-NBD-cholesterol labelled extracellular vesicles. These data indicate that 3-NBD-cholesterol is a versatile cholesterol tracer in different cell models and extracellular vesicles.

1. Introduction

Sterols are essential components in eukaryote cellular membranes where they interact strongly with neighbouring phospholipids [1]. Their overall role in modulation of membrane biophysical properties is conserved across species, although the identity of the dominating sterol may vary, from ergosterol in yeast to cholesterol in mammals [2,3]. Free sterols are unevenly distributed within the cell and the plasma membrane contains most free cellular sterols [3]. Within the membrane sterols are laterally distributed and subdomains enriched in sterol and sphingolipids constitute an important sub-pool of sterols with important biological roles, for example coordination of signalling pathways [4].

Cholesterol is one of the most widely studied membrane components and alterations in membrane cholesterol are known to regulate many different cellular processes, such as cell adhesion and cell migration [5,6]. As a result, cellular cholesterol homeostasis is tightly regulated by a sophisticated biochemical network [7]. Membrane cholesterol is, however, not static and there is a continuous turnover of cholesterol from both the bulk membrane and cholesterol-enriched subdomains [8,9]. Under most circumstances the hydrophobic side chain is buried within the membrane core, with the 3β-hydroxyl group in close apposition to the phospholipid headgroups at the membrane surface, resulting in an approximately vertical configuration of cholesterol [10]. Genetic and pharmacological treatments can influence the retention of cholesterol and its turnover within the membrane [11,12]. Various methods have been used to track cholesterol dynamics, including the use of radioactive sterols, fluorescent sterols and genetically-encoded cholesterol sensors [13–15].

A wide variety of different fluorescent analogs, including intrinsically fluorescent sterols and modified sterols containing a fluorescent moiety based on 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) or boron-dipyrromethene difluoride (BODIPY), have been used to investigate the distribution and dynamics of cholesterol within cells and membrane [16,17]. Typically labelling has been achieved on various positions of the isooctyl side chain of cholesterol and 22-NBD-cholesterol, 25-NBD-cholesterol and 24-BODIPY-cholesterol (also known as TopFluor cholesterol) are all widely used to investigate sterol dynamics [18–20]. However, the introduction of a bulky group on the side chain would be expected to significantly impact properties of the cholesterol and thus the bulk membrane.

Consistent with this prediction, a molecular dynamics study of both 22- and 25-NBD-cholesterol described an aberrant orientation of both...
steroids whereby the side-chain fluorophore was oriented towards the surface of the membrane [20]. These compounds were unable to recapitulate the key features of cholesterol’s behaviour in membranes with the rate of transfer of 22-NBD-cholesterol between liposomes several orders of magnitude greater than tritiated cholesterol [21]. Indeed, the rapid transfer of 22-NBD-cholesterol (order of seconds) was more similar to that of side-chain oxidised oxysterols such as 25-hydroxycholesterol [20, 21]. In addition, early studies of 24-BODIPY-cholesterol indicated that the rate of efflux of this labelled sterol to various extracellular acceptors was significantly increased compared to tritiated cholesterol [22].

A series of cholesterol analogs incorporating the fluorophore label at the C3-hydroxyl position were prepared by Ramirez and collaborators in 2010 [23]. In contrast to the molecules noted above, the NBD fluorophore is attached via an acyl spacer at the C3 position. The orientation of the molecule is similar to that of cholesterol, i.e. the label is located in the polar headgroup region of the membrane and the isooctyl side chain is buried in the membrane core. As initial studies were completed in model membrane systems, we considered it of interest to test the potential of 3-hexanoyl-NBD-cholesterol as a cholesterol tracer for a variety of applications, with a particular interest in its use in relation to measuring uptake of extracellular vesicles.

2. Materials

2.1. Preparation of labelled cells

Washed erythrocytes were prepared from citrate-anticoagulated whole blood obtained from healthy donors, essentially as previously described [24]. Briefly, cells and plasma were separated by centrifugation at 1500×g for 10 min and the packed red cells were washed three times in a five-fold excess of red cell flux buffer (150 mM NaCl, 5 mM NaP, 5 mM glucose, pH 7.4) and stored on ice until required. To inactivate LCAT, autologous plasma was heat-treated at 56 °C for 50 min, followed by centrifugation at 15,000×g for 30 min. The clear central fraction of the plasma was removed for exchange experiments with autologous packed erythrocytes and stored on ice until required. Labelling was achieved by incubating washed, packed erythrocytes (500 μL) with 10 μg of 3-NBD-cholesterol (from a stock solution of 200 μg/mL in DMSO, Cayman Chemical) overnight at 4 °C. Following incubation, labelled cells were washed three times with flux buffer as described above and stored on ice until required. Cells were used within 8 h of labelling and within 24 h of collection.

2.2. Cholesterol exchange assay

Immediately before use, heat-inactivated plasma was diluted 1:1 (v/v) in flux buffer and warmed to 37 °C. For each time point a separate 10 μL aliquot of 3-NBD-cholesterol labelled erythrocytes was transferred to a 1.5 mL microcentrifuge tube, mixed with 90 μL of diluted plasma and incubated for the appropriate duration. At each time point, the sample was removed, rapidly transferred to a pre-chilled microcentrifuge (4 °C) and centrifuged at 21,000×g for 15 s. A 45 μL aliquot of the supernatant was removed and reserved for analysis. Fluorescence intensity was measured on a multi-mode microplate reader (SpectraMax M3, Molecular Device Company) using black-walled microplates and a wavelength of 486 nm for excitation and 525 nm for emission. The total available 3-NBD-cholesterol available for exchange determined by lipid extract of labelled erythrocytes and subsequent fluorescent analysis as above. Results are expressed as a percentage of total available label (mean of three independent experiments and erythrocyte samples, three technical replicates per experiment).

2.3. Detection of labelled cells via flow cytometry

HeLa cells (3.5 × 10^5) were seeded into six well plates and labelled with 3-NBD-cholesterol using the stock solution described above (final concentration of 1 μg/mL). At each time point, cells were washed three times with cold PBS, harvested and analysed by flow cytometry using a BD Accuri C6 (BD Biosciences). Cell-associated fluorescence was estimated using the FL1 channel (emission 530/30 nm).

2.4. Detection of labelled cells by confocal microscopy

HeLa and HMDM cultured on glass bottomed 35 mm dishes were incubated with 1 μg/mL of 3-NBD-cholesterol for 18 h. Following incubation, cells were washed three times with PBS. Live cell images were acquired using the Zeiss LSM 510 inverted confocal microscope (excitation 488 nm).

2.5. Detection of uptake of exogenous 3-NBD-labelled microparticles

Platelet microparticles were isolated from platelet concentrates as described [25]. Briefly, platelet-free plasma was generated from 50 mL of concentrate by three sequential centrifugations at 879×g for ten minutes each. The final supernatant was centrifuged twice at 20,000×g for 60 min to generate a microparticle pellet which was resuspended in a minimal volume of PBS. Microparticle protein concentration was estimated using the bicinchoninic protein assay as per the manufacturer’s guidelines (Abcam, Cambridge, UK). The cellular origin of the microparticle preparations was confirmed by flow cytometry using phycoerythrin labelled anti-CD42 and flow cytometry (Accuri C6 BD). To permit labelling, microparticle pellets (equivalent to 760 μg protein) were resuspended in PBS, mixed with 5 μg of 3-NBD-cholesterol in a total volume of 525 μL and incubated at 4 °C for 48 h. Microparticles were washed twice with cold PBS, resuspended in 550 μL and stored at 4 °C until required. 3-NBD-labelling of microparticles was evaluated using flow cytometry, as described above. To evaluate the uptake by phagocytic cells, 3-NBD-labelled microparticles (final concentration of 69 μg microparticle protein per mL of medium) were added to THP-1 derived macrophage-like cells. At the indicated time points, cells were removed using a cell scraper, washed and resuspended in 100 μL of PBS. The uptake of fluorescent microparticles by live cells was estimated by flow cytometry as described above. Parallel experiments were carried out using a non-phagocytic cell line to evaluate the contribution of exchange of 3-NBD-cholesterol between microparticles and cells to the total detected fluorescence.

2.6. Metabolism of 3-NBD-cholesterol

THP-1 cells were incubated in the presence of 0.285 μg/mL of 3-NBD-cholesterol in DMSO (final concentration of DMSO < 0.5%) for 25 or 72 h. After the incubation time, the cells were collected from the plates, centrifuged to retrieve a cell pellet and the supernatant was retained separately. To extract cellular lipids, the cell pellet was resuspended in 50 μL of deionised water and extracted with 250 μL of 3:2 n-hexane:isopropanol (v/v). Following phase separation, the organic phase was removed and evaporated to dryness. The cellular lipid extract was redissolved in 20 μL of NP40: isopropanol. Cell medium (400 μL) was extracted with 1.2 mL of 3:2 n-hexane:isopropanol (v/v) as described above. Extracts were loaded onto silica TLC plates and separated using a mobile phase of toluene-ethyl acetate (60:40, v/v) as described above. Extracts were visualised and documented using a standard gel doc.

2.7. Statistical analysis

GraphPad Prism (version 7) or Microsoft Excel was used for plots. Curves were fit to the data using non-linear regression (MyCurveFit). Flow Jo 10 was used for flow cytometry data analysis. All data is expressed as mean ± SD or otherwise identified in the figure legends. Where significant differences are shown, the unpaired students T-test was used. Data is available on request.
3. Results

3.1. Exchange of 3-NBD-cholesterol between erythrocytes and plasma lipoproteins

As Fig. 1 shows, transfer of 3-NBD-cholesterol from labelled erythrocytes to heat-inactivated autologous plasma resulted in a time-dependent increase in labelling of the plasma acceptor pool, i.e. lipoproteins. Use of erythrocytes as a model ensures that there is no potential for metabolism of the 3-NBD-cholesterol. In similarity with previous studies using radiolabelled cholesterol, an initial fast phase was followed by a plateau [26,27]. These data are consistent with the behaviour of radiolabelled cholesterol under similar conditions [28].

3.2. Detection of 3-NBD-cholesterol using flow cytometry

Following confirmation that 3-NBD-cholesterol recapitulated cholesterol exchange kinetics in an erythrocyte model, we sought to examine the potential of flow cytometry to detect this cholesterol tracer. As shown in Fig. 2, exposure of HeLa cells to 3-NBD-cholesterol resulted in a clear time-dependent increase in cell labelling, readily observed using flow cytometry (Fig. 2A & B). At 7 h, approximately 80% of exposed cells exhibited at least some positivity, although there was some variability between cells at each timepoint. Confocal fluorescent microscopy revealed similar time-dependent labelling of HeLa cells following incubation with 3-NBD-cholesterol (Fig. 2C). In overnight incubations using HeLa cells and primary human monocyte derived macrophages, a clear accumulation of label in cells was observed (Supplemental Fig. 1).

Fig. 1. Transfer of 3-NBD-cholesterol from erythrocytes to lipoproteins. Individual data points are shown as percent of total label available for transfer. Results are from three independent erythrocyte samples with three technical replicates.

Fig. 2. Detection of 3-NBD-cholesterol labelled cells by flow cytometry. A) Time course of label uptake, B) Representative histograms from flow-cytometry analysis. Cells were labelled and analysed as described in methods. Individual data points are shown as percent of cells labelled. C) Confocal microscopy of time course of uptake of 3-NBD-cholesterol by HeLa cells. Representative samples of two experiments, n = 3 in each case.

Fig. 3. TLC of 3-NBD-cholesterol incubated with THP-1 cells. Lipid extracts were separated as per methods and visualised using a gel-doc. Asterisks indicate faint bands in the cellular fractions. This experiment is from the result of one incubation.
3.3. Metabolism of 3-NBD-cholesterol

TLC analysis of cell and medium extracts obtained following incubation of THP-1 cells in the presence of 3-NBD-cholesterol for 24 or 72 h did not reveal any evidence of metabolism of the added 3-NBD-cholesterol (Fig. 3). The majority of the label was present in the medium although a faint band was observed in the cell extracts (Fig. 3 lanes 2 and 8).

3.4. Uptake of 3-NBD-cholesterol labelled extracellular vesicles

Extracellular vesicles are submicron sized particles which are present in a broad range of biological fluids. We isolated platelet-derived extracellular vesicles and labelled them with 3-NBD-cholesterol (Fig. 3). The majority of the label was present in the medium (Fig. 3 lanes 2 and 8).

4. Discussion

Cholesterol exchange between lipophilic compartments is a fundamental feature of cholesterol homeostasis, whether the exchange is between cells and lipoproteins or between lipophilic binding sites of intracellular proteins. Although radiolabelled cholesterol is considered to be the gold standard for studies of cholesterol transfer, efforts have been ongoing to identify a fluorescent tracer which would obviate the safety issues related to radioactive cholesterol and also open up new experimental paradigms [18]. As noted above, several different cholesterol tracers are in common use. Typically these are labelled on the side chain of the sterol molecule [13,18]. This labelling has significant consequences for the physiochemical properties of the molecule. Sterols containing a fluorophore on the side chain (e.g. 22-NBD-cholesterol) transfer between lipophilic compartments orders of magnitude faster than cholesterol itself and have a strongly tilted orientation in the membrane [21]. In fact, the kinetics of exchange and orientation of these molecules are more similar to that of side-chain oxidised oxysterols [20,21]. This essential aspect is not well recognised in the literature, as typically longer-term incubations (e.g. 24 h) are carried out and differences between the initial rate of the various labelled sterols is blunted. However, the bulky side-chain fluorophore will impact the behaviour of the tracer inside the cell and may lead to erroneous results in the estimation of the ‘exchangeability’ of cholesterol in the cell [29].

In contrast, as there are no substituents on the side chain 3-NBD-cholesterol would be expected to preserve the orientation of cholesterol in the membrane and more faithfully recapitulate the membrane properties of cholesterol. In agreement with this contention, the flux of cholesterol between erythrocytes and acceptor plasma lipoproteins was near identical to that previously observed for radiolabelled cholesterol. These data were very reproducible, with similar results obtained by multiple independent experimenters. In addition to these exchange
studies, 3-NBD-cholesterol as also amenable to detection by flow cytometry and microscopy, significant advantages over classical radio-labelled techniques. Similar patterns and kinetics of labelling were found using the different approaches, supporting the notion that this is a broadly applicable tracer.

A significant obstacle in the field of extracellular vesicles is a strategy which labels vesicles of all cell types. As it is well known that extracellular vesicles contain cholesterol, we hypothesised that 3-NBD-cholesterol would be capable of labelling these vesicles. Our current data shows that 3-NBD-cholesterol labelled vesicles readily label both phagocytic and poorly-phagocytic cell systems. Multiple pathways are believed to be involved in the uptake extracellular vesicles including classical phagocytosis, lipid raft-mediated internalisation, caveolin-mediated and micropinocytosis-based uptake [30,31]. The significantly greater labelling of the phagocytic cells indicates that phagocytosis plays a major role in the uptake of extracellular vesicles, as tracked using 3-NBD-cholesterol. In addition, it is possible that direct physical contact between labelled extracellular vesicles and cellular membranes may lead to an exchange of 3-NBD-cholesterol between them, similar to that observed for erythrocytes. Data from pilot pulse-chase experiments supports this contention with the signal for CD42b lost at a greater rate than that of 3-NBD-cholesterol (results not shown).

Despite these potential limitations it was possible to observe a clear time dependent increase in 3-NBD-cholesterol associated fluorescence in the phagocytic THP-1 macrophage-like cells. To exclude the possibility that the 3-NBD-cholesterol was metabolised (e.g. to a more polarsterol) or that the C3 label was removed, we examined the stability of 3-NBD-cholesterol by thin layer chromatography. There was no apparent metabolism of the label in either differentiated THP-1 cells or human monocyte-derived macrophages, cells known to express high levels of sterol metabolising enzymes (e.g. sterol 27-hydroxylase, cholesteryl ester hydrolase) (results not shown). While it is not possible to exclude the possibility of metabolism by other cells, our initial data indicates that 3-NBD-cholesterol may be metabolically stable in some cell systems of importance for studies of cholesterol homeostasis.

Taken together, these studies indicate that 3-NBD-cholesterol has the potential to be a multimodal tracer of cholesterol with applicability in studies of cholesterol efflux, exchange and as a general marker of extracellular vesicles. Studies are ongoing to explore the utility of 3-NBD-cholesterol in more detail.

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Appendix A. Supplementary data

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